BIOCHEMICAL GENETICS OF HYDROGEN METABOLISM IN *ESCHERICHIA COLI*: PURIFICATION AND CHARACTERIZATION OF HYDROGENASE

By

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LIST OF ABBREVIATIONS

BV.....Benzyl viologen

DEAE.....Diethylaminoethyl cellulose

EDTA.....Ethylenediaminetetraacetic acid

FHL.....Formate hydrogenlyase

HUP.....Hydrogen uptake

MV.....Methyl viologen

PAGE.....Polyacrylamide gel electrophoresis

PEG.....Polyethylene glycol

SDS.....Sodium dodecyl sulfate

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A procedure is described for the purification of membrane-bound hydrogenase from *Escherichia coli*. This procedure uses a non-ionic detergent, precipitating agents, and a series of column chromatography steps to purify the protein to homogeneity. The molecular weight of the protein is determined in the presence and absence of detergent by gel filtration and polyacrylamide gel electrophoresis, under non-denaturing conditions. These results show that the enzyme exists predominantly as a monomer (59,000 d) in the presence of Triton X-100 and as a dimer (124,000 d) in its absence. The subunit molecular weight is 56,000. The enzyme has

4.4 molecules of iron and 4.7 molecules of acid-labile sulfur per 59,000 d protein. The optimum temperture for catalysis of the exchange reaction is 35°C and has a broad pH optimum between pH values of 7.0 and 7.5. The enzyme has an apparent Km of 26.7 mM for oxidized methyl viologen, 7.7 mM for oxidized benzyl viologen, 1.5 mM for reduced methyl viologen, 4.0 mM for reduced benzyl viologen and 1.6 mM for hydrogen. The dimer is twice as active as the monomer. The enzyme is inactivated by air with a half life of 650 minutes. Comparison of the biochemical properties of the pure hydrogenase with the hydrogenase produced by the HUP$^-$ mutants suggests the pure hydrogenase is involved in the hydrogen uptake reaction in E. coli.

S tudy of the regulation of hydrogenase activity in whole cells indicates that the enzyme activity is inducible under anaerobic conditions and maximal levels of hydrogenase activity can be detected within 60 minutes after anaerobic shift. Hydrogenase activity declined rapidly upon the addition of electron acceptors, viz., oxygen and nitrate. The rate of decline of hydrogenase activity due to the addition of nitrate was bi-phasic with an initial half life of 40 minutes and eighty minutes after addition of nitrate, the hydrogenase activity declined with a half life of 10 minutes. This rapid decline in activity was accompanied by the appearance of nitrite in the medium. The half life of hydrogenase activity of the culture exposed to oxygen is 8 minutes.

INTRODUCTION

Since the dawn of civilization, mankind has been in search of an efficient source of energy. It has been shown throughout history that there is a direct correlation between the energy consumption per capita and the standard of living.

Today, the world's population is increasing at an alarming rate. Most of the increase in the population is taking place in the impoverished nations of the world. As this major part of the world's population strives to improve their standard of living, there will be an unprecedented demand on the energy resources of the world. This increase in demand will have far-reaching consequences on the life of every living being on this planet.

As we have already seen, the increased demand for energy in the last couple of decades has sent the price of fossil fuels on a sharp increase. As a result of this, the developing countries are caught in a "no win" situation. To become industrialized, a nation has to have an abundance of energy at its disposal, and to be able to import the energy it needs for industrialization, it has to have the economic resources. Also, the increased demand in energy, world wide, has led to some of the major political crises around the globe.

Political and economic consequences of the increase in energy demand are not the only concerns that we have to address. Due to the increase in the energy demand, one of the questions that we have to ask ourselves is the effect the increase in energy consumption will have on the environment. Most of our energy demand today is met by fossil fuel. It is apparent that the fossil fuel reserves will be eventually depleted, and in any event, the natural environment cannot readily assimilate the by-products of fossil fuel consumption at much higher rates than it does without suffering unacceptable levels of environmental decay. Thus, today more than ever before, we realize the importance of a fuel source that is economical and efficient, has no toxic end products, and can be recycled.

With this bleak outlook for the future in the field of energy, a lot of money and effort have been spent in exploring alternative sources of energy that are efficient and non-polluting. At present, the two most promising areas seem to be solar energy and biomass conversion. Both these fields have the advantage of being present in nature in abundance.

One of the products that can be obtained from both biomass conversion and solar energy is hydrogen. Hydrogen as an energy source of the future has enormous potential. It has the advantage of being a very clean fuel. Unlike the fossil fuels of today, combustion of hydrogen does not yield any of the toxic waste products, viz., SO_2, CO, NO_x etc. Water is the main product and the

water produced is easily recycled in nature. The biggest advantage of using hydrogen as a fuel source of the future is the fact that it can be produced on a large scale using biological systems. Hydrogen is produced by most procaryotic microorganisms (5,119). We can exploit these biological systems and utilize the enormous pool of energy-rich compounds such as lignin, cellulose and cellobiose found in nature. Hydrogen is also produced by cyanobacteria and photosynthetic bacteria (115). During photosynthesis, light energy is used to generate energy-rich compounds such as NADH, NADPH and ATP. The ATP, NADH and NADPH can be used to reduce protons resulting in the evolution of hydrogen. Thus, it is conceivable that some day in the future hydrogen can be produced on a large scale by harnessing solar energy.

However, if we are to exploit hydrogen as the energy of the future we should have a detailed understanding of the role it plays in nature. Biologically, hydrogen is produced by most procaryotic organisms growing in the absence of inorganic electron acceptors such as oxygen or nitrate (40,121). During fermentation, many bacteria use protons as an electron sink, resulting in the evolution of hydrogen. Hydrogen is also consumed by many bacteria as a source of reducing power under appropriate conditions (5,119). During the formation of methane, a widely used fuel, methanogens utilize hydrogen as a source of reductant to reduce CO_2. If we understand

the role of hydrogen gas in these reactions to a greater extent, we will be able to exploit it for our own good.

At the center of the hydrogen metabolism in biological systems lies the enzyme hydrogenase. It catalyzes the primary reaction in hydrogen metabolism, the reversible activation of a hydrogen molecule. Thus it is imperative that we understand the mechanism of catalysis of this basic reaction and its regulation in nature.

The best way to approach the basic questions regarding the mechanism of catalysis and its optimal conditions for catalysis is to study the enzyme after it has been obtained in a pure state. The purified enzyme can also be used to raise antibodies in a mammal. The antibodies can then be used to elucidate the regulation of hydrogenase synthesis, identify the structural gene for the enzyme and also determine the location of hydrogenase in the membrane with respect to other proteins.

Since E. coli possesses hydrogenase it seems logical to examine the role it plays in the cells metabolism. E. coli offers the following advantages: 1) It is a very well studied organism with respect to metabolism; 2) Has established techniques to investigate the biochemistry of the various metabolic reactions and for genetic manipulation of the organism; 3) The ease with which a large number of mutants can be isolated and characterized. A thorough understanding of hydrogen metabolism in one organism may help us better understand the hydrogen metabolism of other micro-organisms.

In Escherichia coli, hydrogenase is known to be a part of the formate hydrogenlyase enzyme complex which is responsible for the evolution of hydrogen gas from formate under anaerobic growth conditions. Hydrogenase is also involved in the growth of E. coli under conditions where hydrogen is the source of reductant, in the presence of a suitable electron acceptor, viz., fumarate. It is not known whether the same enzyme is involved in both the hydrogen evolution and uptake reactions, or whether there are two separate hydrogenases responsible for the two different reactions.

There are a number of reports in the literature concerning hydrogen metabolism in E. coli. Most of the studies have been directed toward characterization of the enzyme hydrogenase, and toward determining the number of hydrogenase proteins in a cell (details are presented in the Literature Review section). However, the number of hydrogenases present in the cell has not yet been established conclusively. Even the reports describing the physical properties of the enzyme provide conflicting molecular weights for the enzyme. Adams and Hall (4) reported a molecular weight of 113,000 for the hydrogenase they purified from E. coli. In 1978, Bernhard and Gottschalk (12), reported a molecular weight of 191,000 for hydrogenase from E. coli. The molecular weight in the latter report was determined by the sucrose density gradient centrifugation and gel filtration of a partially purified preparation. On the other hand, Graham et al. (37), in 1980, reported a molecular weight

of 56,000 for hydrogenase from E. coli. They determined the molecular weight by an indirect method which involved electrophoresis of solubilized membrane vesicles in native PAGE, staining for hydrogenase activity followed by SDS-PAGE of the excised active band from the gel. It should also be stated that both, Adams and Hall (4), and Bernard and Gottschalk (12), used proteases to aid in the solubilization of the enzyme from the membrane. It is possible that the enzyme obtained after protease treatment may not reflect the properties of the native enzyme.

This work describes a scheme for purification of the enzyme hydrogenase to homogeneity without the use of proteolytic agents. The enzyme is characterized with respect to its biochemical and physical properties. The regulation of the enzyme in cultures growing in the presence or absence of electron acceptors such as oxygen and nitrate is also studied.

LITERATURE REVIEW

Production and consumption of hydrogen gas by microorganisms has been known since the turn of the century (44). However, it was only in the 1930's that the enzyme responsible for the evolution of hydrogen gas during bacterial fermentation was identified and the physiology of this process studied (24,41,98,113). During an investigation of a pollution problem in the Great Ouse river, Stephenson and Stickland (98) observed that the microorganisms thriving upon the sugar beet waste dumped into the sluggish river were responsible for the evolution of gases such as hydrogen, carbon dioxide and methane. They also showed that washed cultures of bacterial isolates from the river bed were able to reduce methylene blue in the presence of hydrogen. This observation led them to conclude that the microorganisms possesed an enzyme capable of activating a molecule of hydrogen. This enzyme, which they termed hydrogenase (EC 1.12), catalyses the reversible reaction as represented by the equation

$$H_2 + e^-\text{carrier (oxidised)} \rightleftharpoons 2\,H^+ + e^-\text{carrier (reduced)}$$

Since the initial discovery of hydrogenase, the enzyme has been shown to be present in a diverse group of microorganisms, both bacteria and algae (5,40,119). In vivo, hydrogenase is usually coupled to other electron carriers or is a part of a multienzyme complex, and thus, the enzyme generally catalyzes an "irreversible" reaction, in vivo. However, in the presence of suitable electron donors or acceptors all pure hydrogenase proteins examined so far, catalyze reversible reactions.

Physiological Role of Hydrogenase

The physiological role of hydrogenase in the anaerobic metabolism of microorganisms can be divided into two categories : 1) evolution of hydrogen and 2) consumption of hydrogen.

In the fermentative bacteria, evolution of hydrogen via hydrogenase can be seen as a means of oxidizing the electron carriers reduced during fermentation. This oxidation is required to allow the electron carriers to recycle so that a continuous supply of ATP can be generated by substrate level phosphorylation. For example, in Clostridium pasteurianum, each mole of glucose yields two moles of pyruvate which is further degraded to acetyl-CoA, CO_2 and hydrogen, via pyruvate:ferredoxin oxidoreductase and hydrogenase (104,105,106). Some of the excess NADH generated at the level of glyceraldehyde-3-phosphate dehydrogenase is oxidised to produce

hydrogen by NADH:ferredoxin oxidoreductase and hydrogenase (5,27,59). Thus, the redox balance is maintained in the cell without the need for terminal electron acceptors other than protons. In E. coli pyruvate is metabolized via the pyruvate-formatelyase enzyme complex to formate and acetyl Co-A (56). The formate is further metabolized to CO_2 and H_2 via the formate hydrogenlyase complex of which hydrogenase is an integral part (5,41,42,59).

Under conditions where hydrogen is the only source of reducing power and energy, hydrogenase oxidizes hydrogen. The electrons obtained are utilized to reduce inorganic or organic electron acceptors (5,42,69,76,110). For example, the genus Desulfovibrio possesses the ability to use SO_4^{-2} as a terminal electron acceptor. Electrons obtained from the oxidation of hydrogen are utilized to reduce SO_4^{-2} and yield S^{-2} (6,42). Paracoccus denitrificans has the ability to utilize nitrate as the terminal electron acceptor for the electrons obtained from the oxidation of hydrogen by hydrogenase (96). In methanogens, hydrogen can serve as the sole electron donor for the reduction of CO_2 to methane (12,120). In E. coli, under appropriate conditions, hydrogen can serve as an electron donor to reduce electron acceptors, such as fumarate, nitrate, and oxygen (5,36,69). In all of these reactions, hydrogenase is an essential enzyme and is associated with the membrane and an electron transport chain (69). Therefore, the oxidation of hydrogen not only serves as

a source of electrons but the association of hydrogenase with the membrane helps generate a proton gradient across the membrane.

Hydrogenase also plays a dual role in photosynthetic bacteria. During photosynthesis, the bacteria utilize hydrogen as a source of reductant for CO_2 fixation (5,115). This ability to utilize hydrogen as a source of reductant is mediated by hydrogenase (53). In addition, during dark fermentation, the purple non-sulphur bacteria metabolize pyruvate via a pyruvate formatelyase and a formate hydrogenlyase system analogous to that found in E. coli (33).

Another group of microorganisms that possess hydrogenase is the aerobic hydrogen oxidizing bacteria. These microorganisms are characterized by their ability to grow autotrophically, using hydrogen as the sole electron donor, CO_2 as the carbon source and oxygen as the terminal oxidant (85,89). Some of the microorganisms belonging to this group have two different types of hydrogenases. One of the hydrogenases is soluble in the cytoplasm and reduces NAD directly with hydrogen (82). The other hydrogenase is membrane bound and donates electrons to the respiratory chain which in turn reduces oxygen and thus produces energy for autotrophic growth (84,91,93).

From the examples cited above, it can be seen that hydrogenase plays an important role in the physiology and metabolism of a diverse group of microorganisms.

Methods to Monitor Hydrogenase Activity

In 1930, the classical hydrogenase assay involved the reduction of methylene blue in the presence of hydrogen (98). Today, hydrogenase activity can be monitored by three different methods: 1) hydrogen evolution, 2) hydrogen consumption and 3) the exchange reaction (59).

In the hydrogen evolution reaction, the ability of hydrogenase to reduce protons in the presence of suitable electron donors is monitored. Electron donors such as reduced ferredoxin (12,73,77), cytochrome c3 (77), and reduced viologen dyes (38,58,66,80,102) have been employed. The rate of hydrogen evolution is monitored either manometrically or by using a gas chromatograph or a hydrogen electrode (58,59,80,112).

Conversely, in the hydrogen uptake reaction the ability of hydrogenase to oxidize hydrogen to protons and electrons is monitored. The oxidation of hydrogen requires the presence of suitable electron acceptors. In the presence of such electron acceptors, the rate of the reaction can be monitored either spectrophotometrically, or by using a gas chromatograph or a hydrogen electrode (5,59,100).

In the exchange reaction, hydrogenase activity can be measured by monitoring the exchange between molecular hydrogen and heavy water or between tritium gas and water (22,23,58,59,111).

Farkas et al. (24) were the first to demonstrate an exchange reaction between hydrogen gas and heavy water catalysed by E. coli. The reaction as represented by the equation

$$HD + H_2O \rightleftharpoons HDO + H_2$$

is catalysed by hydrogenase. The rate of the forward reaction can be measured by monitoring the appearance of the isotope in the aqueous phase. In 1963 Gingras et al.(28) introduced a modification of the exchange assay in which tritium gas was used in place of hydrogen and heavy water was replaced by water. In this modified assay as represented by the equation

$$HT + H_2O \rightleftharpoons HTO + H_2$$

hydrogenase activity can be followed by monitoring the accumulation of tritiated water in the aqueous phase.

If the hydrogen evolution or hydrogen consumption reaction is used to determine hydrogenase activity, one has to bear in mind that the rate of the reaction may not be a true reflection of hydrogenase activity. Since hydrogenase is usually associated with other electron transport proteins in the cell, it is possible that the activity of hydrogenase in whole cells or intact membranes monitored using artificial electron acceptors or donors may reflect the

activity of a multienzyme complex, of which hydrogenase is one of the many components. Gitilitz and Krasna (29) did observe this phenomenon. They found that the activity of hydrogenase from Chromatium, measured by reduction of artificial electron carriers decreased during purification as compared to the exchange activity, indicating the presence of various cellular electron carriers in the crude extract that enhanced oxidation/reduction of artificial electron carriers. Also, since the reaction catalysed by hydrogenase is an oxidation/reduction reaction, the redox potential (Eo') of the substrate determines the rate of the reaction. The exchange reaction, on the contrary, is a direct and simple assay for hydrogenase and it is extremely sensitive. Considering these facts, it appears that the exchange reaction is the method of choice to assay for hydrogenase activity.

Properties of the Enzyme

Purification of any protein is immensely simplified if some of the physical properties and special characteristics of the protein are known. Usually, information on the protein from other systems are used as indicators for "do's and "don'ts". There are a number of reports, in the literature, describing the purification of hydrogenase from a diverse group of microorganisms (3,4,12,16,19,29,31,38,51,70,90,92,94,107,116). Review of the

literature indicates that the enzyme hydrogenase is inactive in the presence of oxygen. Thus, it is important to protect the enzyme from oxygen during the purification procedure. Another factor to be taken into consideration is the location of the enzyme. Hydrogenase in the microbial world is found either in the periplasmic space, membrane associated or as a soluble cytoplasmic enzyme. If the hydrogenase is membrane associated, as in the case of E. coli (4,35), one has to incorporate special procedures during the purification procedure.

Membrane proteins have been solubilized using various detergents (32). Hydrogenase has been successfully solubilized from the membrane using detergents, viz., Triton X-100 (3,35,63,71,95,97), and sodium deoxycholate (4,29,71,83). Some researchers have used proteolytic agents, such as trypsin or pancreatin to aid in the solublization of hydrogenase from the membrane (2,4,94,117). However, one has to realize that the use of proteases during the purification procedure may yield a protein that is altered due to proteolytic digestion.

The sensitivity of the enzyme to inactivation by oxygen has been overcome by deoxygenating the buffers with pre-purified nitrogen or argon, adding 1mM sodium dithionite to scavenge any contaminating oxygen and carrying out all procedures in closed vessels under positive pressure of nitrogen, argon or hydrogen (18,43,45,55,75,109).

Most of the other techniques used are the standard techniques used for protein purification, viz., ion exchange chromatography and gel filtration (48,49). In addition to these established procedures many researchers have taken advantage of the hydrophobic nature of the membrane protein and included chromatography on matrices such as Octyl- or Phenyl-Sepharose (46,49,94,108).

Physical Properties of Hydrogenases: Hydrogenase is one of the few anaerobic proteins that has attracted considerable attention in the recent past. One of the first hydrogenases to be purified to homogeneity was from Clostridium pasteurianum (17) and is a well characterized enzyme. Since the time the first hydrogenase was purified, the enzyme has been obtained in pure state from a number of other organisms (5).

All the hydrogenases studied so far can be grouped into three major groups based on their molecular weight and number of subunits. Most of the hydrogenases are made up of a single polypeptide with an apparent molecular weight ranging from 50,000 to 66,000. The second group of hydrogenases range in molecular weight from 89,000 to 101,000. Hydrogenases belonging to the latter group have two subunits. The large subunit has a molecular weight of about 62,000 to 67,000 and the small subunit ranges from 26,000 to 34,000. Hydrogenases studied from two organisms, namely Paracoccus denitrificans and Alcaligenes eutrophus, belong to a third group with

a molecular weight of about 205,000 and consists of 4 subunits (5,92). There are two large subunits and two small subunits. The molecular weight of the large subunits are 63,000 and 67,000 and the molecular weight of the small subunits range from 31,000 to 33,000 (5,92).

There is some variability with respect to the enzyme's sensitivity to oxygen. All of the hydrogenases are inactive in the presence of oxygen (5). However the inactivation is usually reversible. In Clostridium pasteurianum, Desulfovibrio gigas and Alcaligenes eutrophus H16, the enzyme is extremely sensitive to oxygen and irreversibly inactivated (5).

Presence of Metal Ions in Hydrogenase: Hydrogenase is a non-heme iron sulfur protein. The hydrogenases characterized so far have been reported to have varying contents of iron and acid labile sulfur. Most of the active centers contain 4 atoms of Fe and 4 atoms of acid labile S (5).

A number of reports indicate the presence of at least two other metal ions, nickel and copper. Nickel has been shown to be present in the hydrogenases obtained from Rhodospirillum rubrum (3), Chromatium vinosum (7), E. coli (10), Rhodopseudomonas capsulata (20), Methanobacterium thermoautotrophicum (34), Desulfovibrio vulgaris (Hildenborough) (39), Desulfovibrio desulfuricans (61), Desulfovibrio gigas (101), and Vibrio succinogenes (103). Copper has

also been shown to be a part of the hydrogenase derived from Desulfovibrio vulgaris (Hildenborough) (39).

Hydrogenase from Escherichia coli

Hydrogenase from E. coli and its hydrogen metabolism has attracted considerable amount of attention. As indicated above, E. coli is capable of metabolizing formate to yield hydrogen gas and also utilizing hydrogen as a source of reductant under certain growth conditions (5,69,81). However, it is not yet known whether E. coli has two hydrogenases, one for the formate hydrogenlyase (FHL) reaction and the other for the hydrogen uptake (HUP) reaction, or one single hydrogenase protein that is involved in both the reactions. There have been reports in the literature suggesting the presence of multiple hydrogenases in E. coli. Ackrell et al. (1) reported the presence of three hydrogenase species in E. coli. Yamamoto and Ishimoto (118), demonstrated that extracts of E. coli cells grown in different media, either favoring conditions for hydrogen evolution or hydrogen uptake, when subjected to electrophoresis in polyacrylamide gels exhibited bands possessing hydrogenase activity with different electrophoretic properties. More recently, Ballantine and Boxer (10) have reported the existence of two distinct hydrogenases in E. coli. This contention is based on the detection of two immuno-precipitin arcs possessing hydrogenase activity in extracts of cells grown under

anaerobic conditions. The cross-immuno-electrophoresis was performed using Triton X-100 dispersed membranes as antigen. The antiserum from rabbits immunised with E. coli membranes, was used as antibodies. They also demonstrated three distinct bands of hydrogenase activity upon subjecting Triton dispersed E. coli membranes to electrophoresis in non-denaturing polyacrylamide gels. Two of the bands were not detected when the extract was exposed to alkaline pH (pH 10.0), suggesting that one of the hydrogenase was inactivated at this pH. They also found that the hydrogenase resistant to alkaline pH was not easily solubilized from the membrane. Preliminary experiments in our laboratory also suggest the presence of two distinct hydrogenases in E. coli.

Even though the number of hydrogenases in E. coli has not been determined directly, numerous attempts have been made to characterize the hydrogenase(s) from E. coli. In 1950, Joklik (50) made the first attempt to characterize the hydrogenase from E. coli, followed by Gest (26) in 1952. In 1957, Kondo et al. (57) published a procedure for the solublization of hydrogenase using 4% deoxycholate. It was not until 1979 that the first report describing the purification of hydrogenase in E. coli to a high degree of purity was reported. Bernhard and Gottschalk (12) published a report on the purification of the enzyme using a modified method of Kondo et al. (57). Employing trypsin and deoxycholate, they obtained a preparation of the enzyme estimated to be 80% pure. The molecular weight as

determined by gel filtration and density gradient centrifugation was reported to be 191,000. They also reported the enzyme to be irreversibly inactivated in the presence of oxygen with a half life of 36 hours.

In 1979, Adams and Hall (4) reported the purification of hydrogenase from E. coli to homogeneity. Their procedure differed in that they used aerobically grown cells obtained from a comercial source and solubilized the enzyme with sodium deoxycholate and pancreatin. The enzyme, a cytoplasmic membrane-bound protein, is reported to have a molecular weight of 11 3,000 and consists of a dimer of identical subunits. The enzyme, an iron sulphur protein, has 12 Fe and 12 acid labile S atoms per molecule. They reported a half life for hydrogenase of 12 hours under air at room temperature.

In 1980 Graham et al. (37) determined the molecular weight of Hydrogenase from E. coli to be 58,000. The molecular weight was determined by subjecting Triton X-100 solubilized E. coli membranes to Native PAGE and staining the gel for hydrogenase activity. The band possessing hydrogenase activity was then cut from the gel and the protein was eluted and subjected to SDS-PAGE in cylindrical gels. In 1981, however, Graham (35) reported a molecular weight of 63,000. In the latter case the molecular weight was determined using polyacrylamide gel electrophoresis in slab gels. He also reported the enzyme to be a trans-membranous protein.

Genetic studies thus far have not yet identified the structural gene(s) of hydrogenase in E. coli. Pascal and her co-workers (78) reported the isolation of a hydrogenase activity-deficient mutant. However, the mutant also lacked formate dehydrogenase activity, indicating that these mutants could be defective in the formate hydrogenlyase enzyme complex. The mutant strains described by Graham and his co-workers (37) and Krasna (60) also fall into the same category. Glick and his co-workers (30) reported the isolation of a hydrogenase defective mutant, however, analysis of the mutant in our laboratory has indicated that the mutant does possess hydrogenase activity. In 1983, Bock and his co-workers (79) used Mud1(Ap,-lac) insertion mutagenesis to obtain mutants defective in hydrogenase. They were successful in isolating a mutant that lacked hydrogenase activity. Using beta-galactosidase activity as a means of monitoring regulation of synthesis, they showed that the gene affected by the Mud insertion was synthesized only under anaerobic conditions and in the absence of electron acceptors such as nitrate. In 1981, Tait and his co-workers (99) described the isolation of strains of E. coli that lacked hydrogenase activity. All of the mutants defective in hydrogen metabolism are affected only in those genes that are essential for hydrogenase activity. In 1983 Karube and his co-workers (52) reported the isolation of a hydrogenase mutant from E. coli. The mutant strain, which is unable to reduce methyl

viologen as tested by the filter-dye reduction method, was isolated after mutagenesis with N-methyl N'-nitro-N-nitrosoguanidine.

Exploiting a positive selection method to isolate mutants of E. coli defective in its hydrogen metabolism (65), our laboratory has successfully isolated a large number of such mutants. Based on phenotypic characteristics, the mutants have been grouped into two distinct classes. One of these classes, which is defective in hydrogen uptake, did produce an active formate hydrogenlyase, has a lesion near 65 minutes in the E. coli chromosome (genetic map (9) and is 76% co-transducible with metC (65). The other class of mutants which lack hydrogenase activity have lesions between srl and cys operons (58 and 59 min, respectively) on the chromosome. Based on fine structure analysis of this region, the latter class of mutants have been further subdivided into two sub-groups. These two sub-groups belong to two distinct operons. Genes from both of these operons are essential to produce an active hydrogenase in the cell. Segments of DNA from wild type E. coli containing both of these operons have been cloned (88). Further genetic studies of the region have shown that there are at least four distinct genes responsible for the production of an active hydrogenase in the cell (personal communication, Sankar, P. and Lee, J.H.).

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacterial strains used in this study are listed in Table 3-1. Bacterial cultures for each experiment were grown as described for each individual experiment, in the Results Section.

Chemicals

All the chemicals used were of analytical grade and were obtained from Fisher Scientific Company, Pittsburgh, PA, or Sigma Chemical Co., St. Louis, MO.

Media

The mineral base for the minimal medium used to cultivate E.coli had the following composition: (grams/liter) Na_2HPO_4, 6.25; KH_2PO_4, 0.75; NaCl, 2.00; $(NH_4)_2SO_4$, 1.00; $MgSO_4.7H_2O$, 0.20; $FeSO_4.7H_2O$, 0.010; $NaMoO_4.2H_2O$, 0.010; Na_2SeO_3, 0.000263. The pH of the medium was 7.5. The carbon source was glucose and was supplied at a concentration as described for particular experiments.

Table 3-1. Bacterial strains used in this study.

Strain	Genotype or Phenotype	Source
K-10	Hfr PO2A relA1 pit-10 tonA22 $T2^r$ + spoT	L. Csonka
JC10244	cysC43 alaS3 srl-300::Tn10 thr1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 supE44	L. Csonka
SE-8	thi-1 leu-6 suc-10 bioA2(?) galT27 rpsL129 chlC3 λ^- hup101::Tn10	Laboratory Collection (65)
SE-49	Same as JC 10244 but $alaS^+$ recA56 and hup103	Laboratory Collection (65)
SD 7	gal-25, λ^-, topA10, pyrF287, fnr-1, rpsL195, gyrB226, iclR7 and trp72	B. Bachmann CGSG 6335

* CGSC, Coli Genetic Stock Center.

Luria broth (LB) medium had the following composition: (grams/liter) Bacto tryptone, 10.00; Bacto yeast extract, 5.00; NaCl, 10.00; The pH of the medium was 7.0. All solid media contained 15 grams of agar per liter of medium.

Enzyme Assays

Hydrogen Uptake: Hydrogen uptake activity was measured at room temperature using a Spectronic 710 spectrophotometer. The reaction was carried out in a 12 x 75 mm test tube. A 2.5 ml reaction mixture contained 2.3 ml of 10 mM phosphate buffer, pH 7.0, and the electron acceptor at concentrations described for each experiment. The tubes were capped with serum stoppers, evacuated and filled with hydrogen several times. The reaction was started by adding hydrogenase, 0.2 ml, to attain a final protein concentration of 12.0 microgram/ml. The contents were mixed and the reduction of the electron acceptor was measured at the appropriate wavelength. The electron acceptors used and their extinction coefficients were : methyl viologen, 12,000 M^{-1}. cm^{-1} at 600 nm; benzyl viologen, 7,780 M^{-1}. cm^{-1} at 550 nm; neutral red, 7600 M^{-1} at 450 nm; methylene blue, 7000 M^{-1} at 601 nm; phenosafranin, 1150 M^{-1} at 400 nm; and potassium ferricyanide 13200 M^{-1} at 405 nm (The Merck Index, tenth edition, ed. M. Windholz, Merck and Co., Rahway, N.J.).

Hydrogen Evolution: The assay to measure hydrogen evolution from reduced viologen dyes was carried out in 9.0 ml serum vials, at 23°C. Eight hundred and seventy five microliters of 10 mM phosphate buffer, pH 7.0, containing either methyl viologen or Benzyl Viologen concentrations described for each experiment was placed in serum vials. The vials were capped with serum stoppers, evacuated, and filled with nitrogen, six times. The reaction was started by adding 75 microliters of hydrogenase, to attain a final protein concentration of 2.0 microgram/ml. To reduce the viologen dye, 50 microliters of sodium dithionite was added anaerobically, with a syringe, to attain a final concentration of 50 mM. The final reaction volume was 1.0 ml. The rate of hydrogen evolution was monitored using a Varian Model 910 gas chromatograph.

Tritium Exchange : The reaction was carried out in a 12 x 75 mm test tube. Ninety microlitres of 10 mM phosphate buffer, pH 7.0, was placed in a tube (12 x 75 mm), and the tube was sealed with a serum stopper. The 5.1 ml gas phase was replaced with helium by evacuating the tube and filling it with helium. The procedure was repeated six times. Hydrogenase was added to a final concentration of 0.25 microgram/ml, and 0.1 ml of sodium dithionite, pH 7.0, to attain a final concentration of 1.0 mM. Eight hundred microliters of hydrogen gas was added to each of the assay tubes with a syringe. Tritium gas (11.2 mCi/mmol; New England Nuclear Corp. Boston, MA.) was added (25

microliters) to a final concentration of 0.55 micro-curie per assay as a means to monitor the exchange reaction. After 1 hour of incubation at 37°C, the serum stopper was removed and the tritium gas was vented in a fume hood for 10 min. To 100 microliters of the assay mixture, 2.5 ml of a water-based scintillant was added. Tritiated water present in the 100 microliter fraction was determined with the aid of a scintillation counter. The assay used to monitor hydrogenase activity during the purification procedure was the same, except the sample assayed was 100 microliters in a final assay volume of 200 microliters, tritium gas was added to a final concentration of 0.22 micro-curie per assay and no hydrogen gas was added.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis under non-denaturing conditions was performed as described by Davis et al. (21). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Laemmli (62). The gels were run as either tube gels, or slab gels. The dimensions of the tube gels were 0.6 cm x 8.8 cm. The volume of the separatory gel used was 2.2 ml. The dimensions of the slab gels were 17 x 14.5 x 0.15 cm. The volume of the separatory gel was 30 ml. Location of hydrogenase, after electrophoresis in non-denaturing gels was determined by incubating the gel in 10 mM phosphate buffer, pH 7.0, containing benzyl viologen at a final

concentration of 0.2% and under an atmosphere of hydrogen. The reduced benzyl viologen, which is auto-oxidizable, was made to further react with 2,3,5-triphenyl tetrazolium chloride to produce a bright red permanent band of reduced formazan. The gels were stained for protein with either the silver stain method described by Morrissey (72), or with the aid of coomassie blue R-250 as described by Wilson (114). The molecular weight standards used for the determination of the molecular weight of hydrogenase using SDS-PAGE were obtained from Sigma Chemical Co., St. Louis, MO, and consisted of alpha-lactalbumin, 14,200; trypsin inhibitor, soybean, 20,100; trypsinogen, PMSF treated, 24,000; carbonic anhydrase, bovine erythrocytes, 29,000; glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle, 36,000; albumin, egg, 45,000; and albumin, bovine, 66,000.

Molecular Weight Determination

Gel Filtration: The Sephadex G-200 used for gel filtration was swollen by incubating the beads in deionised water (15 g of beads per liter of water) for 72 hours at room temperature. After 72 hours a column of 1.8 x 48 cm was packed with the swollen Sephadex G-200. The column was equilibrated with 10 mM phosphate buffer, pH 7.0, (Triton X-100 concentration was 0.3%, if present). The column was maintained at a flow rate of 11 ml/hr at $4^{o}C$ for 16 hours before use.

Blue Dextran (MW = 2,000,000) was used to determine the void volume (Vo) of the column. The proteins used for generating a molecular weight calibration curve for the Sephadex G-200 column were obtained from Sigma Chemical Co. and consisted of cytochrome c, horse heart, 12,400; carbonic anhydrase, bovine erythrocytes, 29,000; albumin, bovine serum, 66,000; alcohol dehydrogenase, yeast, 150,000 and beta-amylase, sweet potato, 200,000. The proteins were dissolved in the equilibration buffer at the following concentrations: albumin, 10 mg/ml; alcohol dehydrogenase, 5 mg/ml; beta-amylase, 4 mg/ml; carbonic anhydrase, 3 mg/ml and cytochrome c, 2 mg/ml. The sample volume in all cases was maintained at 1.5 ml. Fractions of 0.93 ml were collected. The calibration curve for the column was generated by plotting the log of the molecular weight of the standard proteins against the ratio of their elution volumes and the void volume for the column. Hydrogenase was loaded at a final concentration of 2.6 micrograms/ml and its elution profile was determined by assaying the fractions collected, for tritium exchange activity. The molecular weight of hydrogenase was determined from the calibration curve.

Native PAGE: To determine the molecular weight of hydrogenase, the enzyme was subjected to electrophoresis in the presence of various concentrations of acrylamide (47). Triton X-100, when present, was incorporated at a final concentration of 0.3%. The electrophoresis was performed in tube gels prepared as described above. After

polymerization, the tube gels were subjected to electrophoresis for 1 hour at 1 mA/gel. After the initial run, samples were loaded and run at 1 mA/gel for the first hour and then the current was increased to 2 mA/gel. The sample volume was 100 microliters, in all cases. The molecular weight standards used were obtained from Sigma Chemical Co. and consisted of alpha-lactalbumin, bovine serum, 14,200; carbonic anhydrase, bovine erythrocytes, 29,000; albumin, chicken egg, 45,000; albumin, bovine serum, 66,000 (monomer); 132,000 (dimer); urease, jack bean, 240,000 (dimer); 480,000 (tetramer). Hydrogenase bands were detected by staining the gels for hydrogenase activity after electrophoresis. The relative migration of the molecular weight standard proteins was determined by staining the gels for protein using the coomassie blue method as described by Wilson (114). A Ferguson plot (25) was generated using the Rf values obtained for the molecular weight standard proteins for each experiment. The molecular weight of hydrogenase was obtained from the Ferguson plot generated for each experiment.

Iron And Acid Labile Sulfide Determination

Total iron was determined by the ortho phenanthroline method as described by Lovenberg et al. (68). Acid labile Sulfide was determined by the method of King and Morris (54).

Protein Determination

Protein was determined using Coomassie Blue G-250 as described by Bradford (15). Albumin, Bovine Serum was used as the standard.

Removal of Triton X-100

Triton X-100 was removed from a sample in two steps. Initially, the Triton X-100 concentration was lowered by dialysing the protein sample against a 100-fold excess of an appropriate buffer, at $4^{o}C$, for 4 hours. After dialysis the residual amount of Triton X-100 was removed by incubating the sample with Bio-Beads SM-2 (20% w/v) on a rocking platform at $4^{o}C$ for 2 hours. The sample was separated from the Bio-Beads SM-2 by centrifugation at 5,000 rpm for 5 min.at $4^{o}C$.

Temperature Profile

Tritium exchange assay was used to determine the temperature profile for hydrogenase activity. The assay was performed as described earlier, both, in the presence and absence of Triton X-100. The only difference was the incubation temperature. The reaction vials were incubated in water baths maintained at 15, 20, 25, 30, 35, 40, 45,and 50 degrees centigrade for one hour.

pH Profile

Tritium exchange assay was used to determine the pH profile for hydrogenase activity. The assay was performed as described earlier. However, 10 mM phosphate buffer was replaced by one of the following buffers present at a final concentration of 100 mM: Tris, pH, 9.5, 9.0, 8.5, 8.0, and 7.5; piperazine-N,N'-bis[2-ethane-sulfonic acid] (PIPES), pH, 8.0, 7.5, and 7.0; and phosphate, pH, 7.5, 7.0, 6.5, 6.0 and 5.5.

Effect of Oxygen on Purified Hydrogenase

To determine the effect of oxygen on purified hydrogenase, 1.0 ml of the enzyme (at a concentration of 2.6 microgram/ml, in 10 mM phosphate buffer, pH 7.0) was placed in a 12 x 75 mm tube. The gas phase in one of the tube was air whereas the gas phase of the control tube was hydrogen. The tubes were rocked (20 oscillations/min.) on a rocking table at room temperature. Samples were withdrawn at various time intervals to determine hydrogenase activity by monitoring the tritium exchange reaction as mentioned above.

Regulation of Hydrogenase

Induction of Hydrogenase: To study the induction of hydrogenase activity in E. coli, strain K-10 was used. The culture used as the

inoculum for the experiment was grown aerobically in 10 ml of LB + 1.5% glucose at 37°C. The culture was grown to an optical density of 0.170 at 420 nm. At this stage, the culture was used to inoculate 40 ml of LB + 1.5% glucose medium in a 70 ml serum bottle. The inoculum size was 25% of the final culture volume. The culture bottle was capped with a serum stopper and flushed with nitrogen. The gas phase in the culture vessel was replaced with nitrogen and the bottle was incubated at 37°C. Every twenty minutes, samples were withdrawn to monitor growth, by measuring the optical density at 420 nm in a spectrophotometer (Bausch and Lomb, Spectronic 710), and the hydrogenase activity by assaying for tritium exchange activity.

Effect of Oxygen on Hydrogenase Activity in Whole Cells: To study the effect of oxygen on hydrogenase activity in whole cells, a culture of E. coli fully induced for hydrogenase was used. E. coli K-10 was grown overnight in LB +1.5% glucose at 37°C under an atmosphere of nitrogen. These cells were used to inoculate 40 ml of LB + 1.5% glucose in two 70 ml serum bottles. The serum bottles were capped with rubber stoppers and the gas phase replaced with nitrogen. The medium was warmed to 37°C and inoculated anaerobically with a syringe with the overnight grown culture. The inoculum size was 1% of the final culture volume. The culture was incubated at 37°C. After 130 minutes, one of the culture bottles was opened and 35 ml of the culture was transferred aseptically to a 500 ml flask and incubated

at 37°C under aerobic conditions shaking at 200 rpm. Samples were withdrawn at various time intervals to monitor growth by measuring the optical density at 420 nm and monitoring the hydrogenase activity by assaying for the tritium exchange reaction.

Effect of Nitrate on Hydrogenase Activity in Whole Cells: To study the effect of nitrate on hydrogenase activity in whole cells, E. coli cells fully induced for hydrogenase activity were exposed to nitrate. E. coli strain K-10 was grown overnight in LB + 1.5% glucose under an atmosphere of nitrogen. This culture was used to inoculate 40 ml of LB + 1.5% glucose in two 70 ml serum bottles. The contents of the bottles were flushed with nitrogen and the gas phase was replaced with nitrogen. The pre-warmed medium was inoculated anaerobically with a syringe. The inoculum size was 1% of the final culture volume. The cultures were incubated at 37°C. After 170 minutes, sodium nitrate was added to one of the cultures anaerobically, with a syringe, to a final concentration of 11.76 mM. Samples were withdrawn periodically to monitor growth by measuring the optical density at 420 nm, and hydrogenase activity by assaying for tritium exchange activity. Accumulation of nitrite in the medium was also determined as described by Van'T Reit et al. (110).

RESULTS

Purification of Hydrogenase

Hydrogenase was purified from a prototrophic strain of Escherichia coli K-12 (strain K-10). The details of the purification procedure and the properties of the enzyme are presented below and in Table 4-1.

Growth of the Cells: Escherichia coli, strain K-10, was grown anaerobically in one liter fleakers filled to the top with LB containing 1.5% glucose and incubated overnight at 37°C. These cells were used to inoculate 15 liter carboys containing minimal medium supplemented with glucose at a final concentration of 3% and casamino acids at a final concentration of 0.1%. The inoculum consisted of 10% of the final culture volume. The carboys were filled to the top (anaerobic growth) and incubated at room temperature for 8 hours. During the later stage of incubation, visible gas production could be observed. The cells were harvested using a De Lavall separator, at room temperature.

Cell Lysis: One hundred and eighteen grams of wet cell paste was suspended in 750 ml of 0.1 M phosphate buffer, pH 7.0, containing

0.01M K-EDTA. The cells were lysed, using lysozyme, egg white (Sigma). A freshly prepared stock solution of Lysozyme was added to the cell suspension to attain a final concentration of 100 microgram/ml. The suspension was incubated at 37°C, shaking at 200 rpm, for 1 hour. The resulting cell lysis increased the viscosity of the suspension. The viscosity was reduced by adding deoxyribonuclease-I, 100 microgram/ml, ribonuclease-A, 100 microgram/ml and $MgCl_2.6H_2O$ to a final concentration 10 mM. The extract was incubated further for 1 hour in a 37°C shaker, mixing at 200 rpm. The extract was centrifuged at 12,000xg for 10 minutes at 4°C, to remove cell debris. The supernatant containing membrane vesicles which had 83,483 units of hydrogenase activity (micromoles of ^3H_2O produced/mg protein. hour) was used for further purification of the enzyme, as described below.

Isolation and Solubilization of Membranes: Since hydrogenase in E. coli is associated with the membrane (4,35), the next step was to separate the membrane vesicles from other soluble proteins. To obtain the membrane vesicles, the extract was centrifuged at 100,000xg in a swinging bucket rotor for 1 hour, at 4°C (all of the procedures henceforth were performed at 4°C and all the buffers were incorporated with sodium dithionite at a final concentration of 1mM, unless indicated otherwise). The pellet containing the membrane vesicles was resuspended in 1,100 ml of 0.1 M phosphate buffer, pH

7.0. Approximately 32% of the hydrogenase activity was recovered in the membrane pellet. To solubilize hydrogenase from the membrane, Triton X-100, a non-ionic detergent, was added to a final concentration of 1.0% and the extract was incubated for 1 hour with gentle rocking. The Triton X-100 solubilized membrane fraction was centrifuged at 100,000xg for 1 hour to remove the non-solubilized membrane vesicles. The supernatant, containing the solubilized membrane proteins, including hydrogenase, was collected. Approximately 55% of the hydrogenase activity present in the membranes was solubilized.

Enrichment of Hydrogenase: To remove some of the lipo-protein complexes that interfere with the purification of the enzyme, polyethylene glycol (PEG) was used. Solid polyethylene glycol 6,000 (recently renamed as PEG 8,000, Sigma Chemical Co., St. Louis, MO) was added to the solubilized membrane protein fraction, to a final concentration of 35% and incubated for 1 hour with gentle mixing. The extract was centrifuged at 12,000xg for 30 minutes. The pellet which contained the hydrogenase activity was resuspended in 500 ml of 0.1 M phosphate buffer, pH,7.0. The hydrogenase apparent specific activity increased to 6.34 units from 4.19 units. Further enrichment of hydrogenase was achieved by ammonium sulfate fractionation. Solid $(NH_4)_2SO_4$ was added to the resuspended pellet to reach a final concentration of 25% saturation. The mixture was incubated for 1

hour with gentle mixing. The proteins that precipitated were removed by centrifugation at 12,000xg for 1 hour. The supernatant which contained the hydrogenase activity was collected. Solid $(NH4)_2SO_4$ was added to the supernatant to attain a final concentration of 60% and incubated for 1 hour. The precipitated proteins were obtained by centrifugation, at 12,000xg for 1 hour. The pellet, which contained hydrogenase activity was resuspended in 200 ml of 0.01 M Tricine buffer, pH 8.0 containing NaCl at a final concentration of 130 mM and Triton X-100 at a final concentration of 1.0%. The resuspended pellet was dialyzed against 6 liters of the same buffer for 6 hours. The dialysis procedure was repeated one more time. This ammonium sulfate enrichment procedure resulted in a 4.5 fold purification of the enzyme without loss of total activity.

DEAE-Cellulose Chromatography: The enzyme was further purified by loading the dialyzed extract on a DEAE-cellulose column (2.8 x 90 cm.), equilibrated with 0.01 M Tricine buffer, pH 8.0, containing NaCl at a final concentration of 130 mM and Triton X-100 at a final concentration of 0.3% (equilibration buffer) and the flow ratemaintained at 45 ml/hr. The column was washed with 600 ml of the equilibration buffer. Hydrogenase was eluted with a linear gradient of 150mM - 225 mM NaCl in a volume of two liters. Five milliliter fractions were collected and assayed for hydrogenase activity as described in the Materials and Methods section. Only the fractions

containing high hydrogenase activity (> 35,000 cpm per assay) were pooled together and dialyzed against 12 liters of 10 mM phosphate buffer, pH 7.0, containing 100 mM $(NH4)_2SO_4$, to remove the bulk of Triton X-100. The protein fraction was dialysed twice against 6 liters of buffer for 6 hours. After dialysis, the remaining traces of Triton X-100 were removed by adding Bio-Beads SM-2 (Bio-Rad) to the extract and incubating the mixture for 2 hours, with gentle mixing. As a result of DEAE-cellulose chromatography, hydrogenase apparent specific activity increased by a factor of eight, although the recovery was less than 20%.

Octyl-Sepharose Chromatography : To remove most of the hydrophilic, and some of the hydrophobic protein contaminants, an Octyl-Sepharose column (2.8 x 40 cm.)was used. The column, fitted with a reverse flow adaptor and maintained at a flow rate of 25 ml/hour was equilibrated with 100 mM $(NH_4)_2SO_4$ in 10 mM phosphate buffer, pH 7.0 (equilibration buffer). The partially purified hydrogenase was applied to the column and the column was washed with 150 ml of the equilibration buffer. Hydrogenase was eluted with a 500 ml linear gradient containing, initially, the equilibration buffer and finally, sodium deoxycholate (0.50 % w/v) and Triton X-100 (0.05 % v/v) in 1 mM phosphate buffer. Two and one half milliliter fractions were collected. The fractions were assayed for hydrogenase activity as described in the Materials and Methods section. Hydrogenase eluted

as a single peak at 0.225% Sodium Deoxycholate and 0.0225% Triton X-100. Fractions containing hydrogenase activity (> 45,000 cpm per assay) were pooled together. Even though hydrogenase was enriched only by a factor of 1.03 during this step, it was important to include this step in the purification procedure, to separate the hydrogenase from some of the other hydrophobic contaminants which interfered with further purification. The pooled fractions were dialyzed against 12 liters of 25 mM Histidine HCl, pH 5.5, containing 0.3% Triton X-100. The dialysis was performed in two steps against 6 liters of buffer for 6 hours each time.

Chromatofocussing: Further purification of the enzyme was achieved by taking advantage of the fact that each protein has its own unique iso-electric point. Chromatofocussing achieves separation of proteins based on their isoelectric points. The dialyzed protein solution containing hydrogenase was applied to a column (1.8 x 45 cm) of Poly Buffer Exchanger (Pharmacia) equilibrated with 25 mM Histidine HCl, pH 5.5 containing 0.3% Triton X-100 and maintained at a flow rate of 20 ml/hour. Hydrogenase activity was eluted by passing 1,200 ml of Polybuffer 74 adjusted to pH 4.0 with HCl and supplemented with 0.3% Triton X-100, through the column. Two ml fractions were collected. The fractions were assayed for hydrogenase activity as described in the Materials and Methods section. Hydrogenase activity eluted as a sharp peak at pH 4.4. Fractions

containing hydrogenase activity, (>23,000 cpm per assay), were pooled together and dialyzed against 6 liters of 50 mM NaCl in 10 mM phosphate buffer, pH 7.0, containing 0.3% Triton X-100. Chromatofocussing resulted in a 4 fold enrichment of hydrogenase activity.

DEAE-Cellulose Chromatography: To purify the hydrogenase to homogeneity, the dialyzed sample obtained after chromatofocussing was applied to a DEAE-cellulose column (1.2 x 30 cm). The column was equilibrated with 50 mM NaCl in 10 mM phosphate buffer, pH 7.0, containing 0.3% Triton X-100 (equilibration buffer) and maintained at a flow rate of 15 ml/hour. The column was washed with 100 ml of the equilibration buffer. Hydrogenase was eluted with a 500 ml linear gradient of 50-125 mM NaCl in 10 mM phosphate buffer, pH 7.0, containing 0.3% Triton X-100. Two ml fractions were collected and assayed for hydrogenase activity. Hydrogenase eluted at approximately 90 mM NaCl. Fractions containing hydrogenase activity were analysed for purity, using 7.5% non-denaturing and 12% SDS-PAGE. The gels were stained for protein using the silver stain method. Fractions containing pure hydrogenase, based on SDS-PAGE, were pooled together and concentrated by ultrafiltration using an Amicon PM-10 membrane ultrafilter. The concentrated sample was also checked for purity by subjecting the sample to electrophoresis as mentioned above. The hydrogenase sample subjected to electrophoresis under

non-denaturing conditions was also stained for hydrogenase activity as described in the Materials and Methods section. Results from native PAGE (Figure 4-1) showed a single band, when stained for protein using the silver stain method. The Rf of the protein band was comparable to the Rf of a protein possessing hydrogenase activity. Figure 4-2 presents the results obtained after the purified enzyme was subjected to SDS-PAGE and the gel stained for protein. A single protein band was detected with a molecular weight of 56,000. The procedure described above yielded a protein which is enriched for by a factor of 690 as compared to its presence in the crude extract. The final yield is 1.43%.

Molecular Weight Determination

To determine the molecular weight of the native enzyme, two different methods were used, namely, gel filtration and native polyacrylamide gel electrophoresis. Also, since the enzyme was solubilized using Triton X-100, and the detergent was present during the purification procedure, the molecular weight was determined, both in the presence and absence of Triton X-100.

Table 4-1. Purification of Hydrogenase.

	Volume (ml)	Protein (mg/ml)	Total Protein (mg/ml)	Apparent Specific* Activity	Total * Activity	Recovery (%)
Extract	724	48.45	35,077	2.38	83,483	100.00
Membrane	1318	12.42	16,369	4.16	68,095	81.57
Solub. Membrane+	1175	7.64	8,977	4.19	37,524	44.95
PEG Pellet	545	5.35	2,921	6.34	18,519	22.18
25% $(NH_4)_2SO_4$ Supernatant	598	4.98	2,906	5.98	17,378	20.82
60% $(NH_4)_2SO_4$ pellet	228	2.85	650	28.72	18,662	22.35

Table 4-1. Continued

	Volume (ml)	Protein (mg/ml)	Total Protein (mg/ml)	Apparent Specific* Activity	Total* Activity	Recovery (%)
DEAE-Cellulose pH 8.0	108	0.43	15.44	219.43	3,388	4.06
Octyl-Sepharose CL-6B	84	0.125	10.584	225.68	2,389	2.86
Chromato-focussing	39	0.086	3.354	843.00	2,827	2.39
DEAE-Cellulose pH 7.0	28	0.026	0.728	1642.00	1,195	1.43

* micromoles of $^{3}H_2O$ produced /mg protein. hr.
+ Solubilized with Triton X-100.

Figure 4-1. Polyacrylamide gel electrophoresis of purified hydrogenase under non-denaturing conditions.
A, Gel stained for protein using the silver stain method;
B, Gel stained for hydrogenase activity as described in the Materials and Methods section.
Amount of protein added to each lane was 2.6 micrograms


A
B

Figure 4-2. SDS-Polyacrylamide gel electrophoresis of purified hydrogenase.
Lane A, purified hydrogenase, (2.0 micrograms);
Lane B, Molecular Weight standard proteins.
Gel stained for protein using the coomassie blue method.


A
B

Gel Filtration in the Presence of 0.3% Triton X-100: To determine the molecular weight of hydrogenase by gel filtration, a Sephadex G-200 column (2.6 cm x 48 cm) was used at 4°C. The column was equilibrated with 10 mM phosphate buffer, pH 7.0, containing 0.3% Triton X-100 and maintained at a flow rate of 11 ml/hr. The void volume (Vo) of the column was determined using blue dextran (MW=2,000,000). A calibration curve for the column was generated with proteins of known molecular weights as described in the Materials and Methods section. Table 4-2 lists the void volume (Vo) of the column, the elution volumes (Ve) of the molecular weight standard proteins and the ratio of Ve/Vo for each of the proteins. To determine the molecular weight of hydrogenase, 1.5 ml of pure hydrogenase (2.6 microgram/ml) in 10 mM phosphate buffer, pH 7.0 + 0.3% Triton X-100 was loaded on the column. The elution of hydrogenase was monitored by assaying the different fractions collected (0.93 ml) for tritium exchange activity. The elution profile of hydrogenase from a representative experiment is presented in Figure 4-3. Hydrogenase activity was detected in two major peaks. The molecular weight of hydrogenase from each peak was determined using the caliberation curve (Figure 4-4). Based on three independent determinations, 22% (+/- 6%) of the activity loaded on the column was detected in peak I and 68% (+/- 9%) of the activity eluted in peak II. Hydrogenase in peak I had a molecular weight of 133,000 (+/- 6,000) and the hydrogenase in peak II had a molecular weight of 62,500 (+/- 4,000).

Table 4-2. Elution pattern of hydrogenase and the molecular weight standard proteins in Sephadex G-200 (2.6 cm x 48 cm column) in the presence and absence of Triton X-100 (0.3%).

Protein	Molecular Weight	+Triton X-100 Elution Volume in ml.	+Triton X-100 Ve/Vo	- Triton X-100 Elution Volume in ml.	- Triton X-100 Ve/Vo
Blue Dextran	2,000,000	35.65		35.05	
Beta-amylase	200,000	50.84	1.43	50.10	1.41
Alcohol dehydrogenase	150,000	54.25	1.52	53.46	1.50
Albumin, Bovine Serum	66,000	63.82	1.79	60.09	1.69
Carbonic anhydrase	29,000	77.50	2.17	74.92	2.10
Cytochrome c	14,200	89.90	2.52	88.35	2.48
Hydrogenase peak I (+Triton) (-Triton)	133,000* 125,000*	67.41	1.89	66.60	1.90
Hydrogenase peak II (+Triton) (-Triton)	62,500* 58,000*	57.18	1.60	56.12	1.60

* Calculated value.

Figure 4-3. Elution profile of hydrogenase in Sephadex G-200 (2.6 cm x 48 cm column) in the presence of 0.3% Triton X-100.

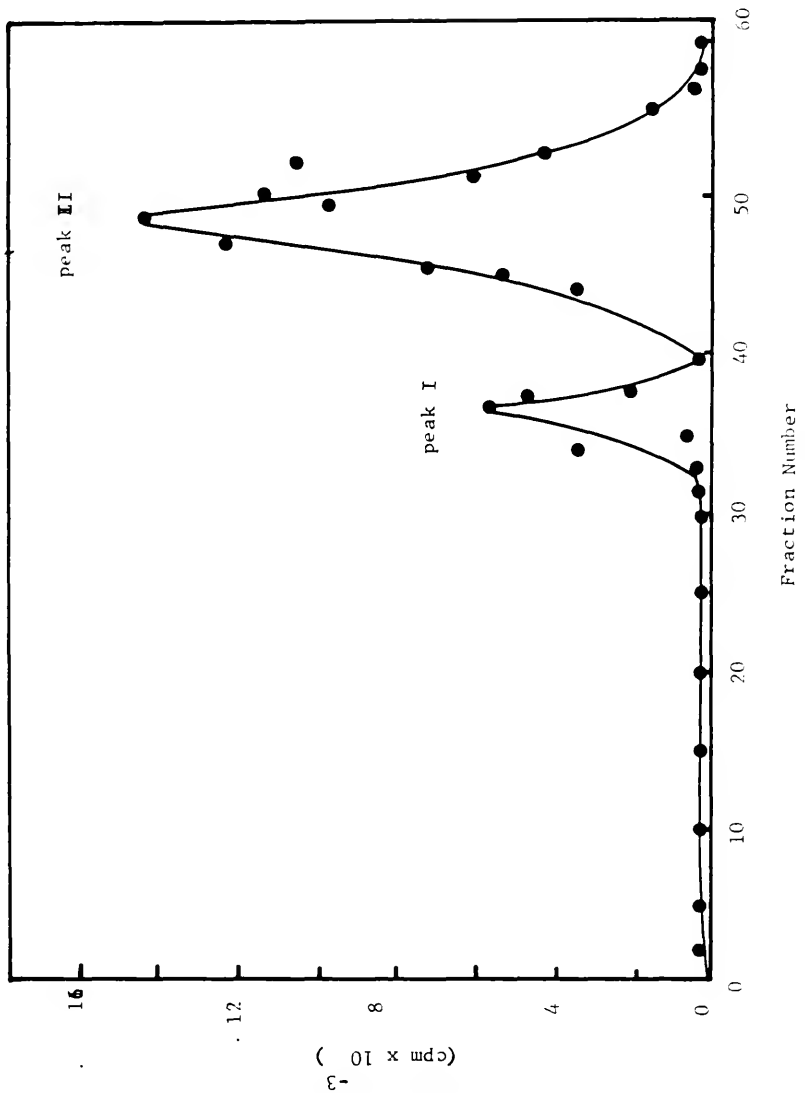
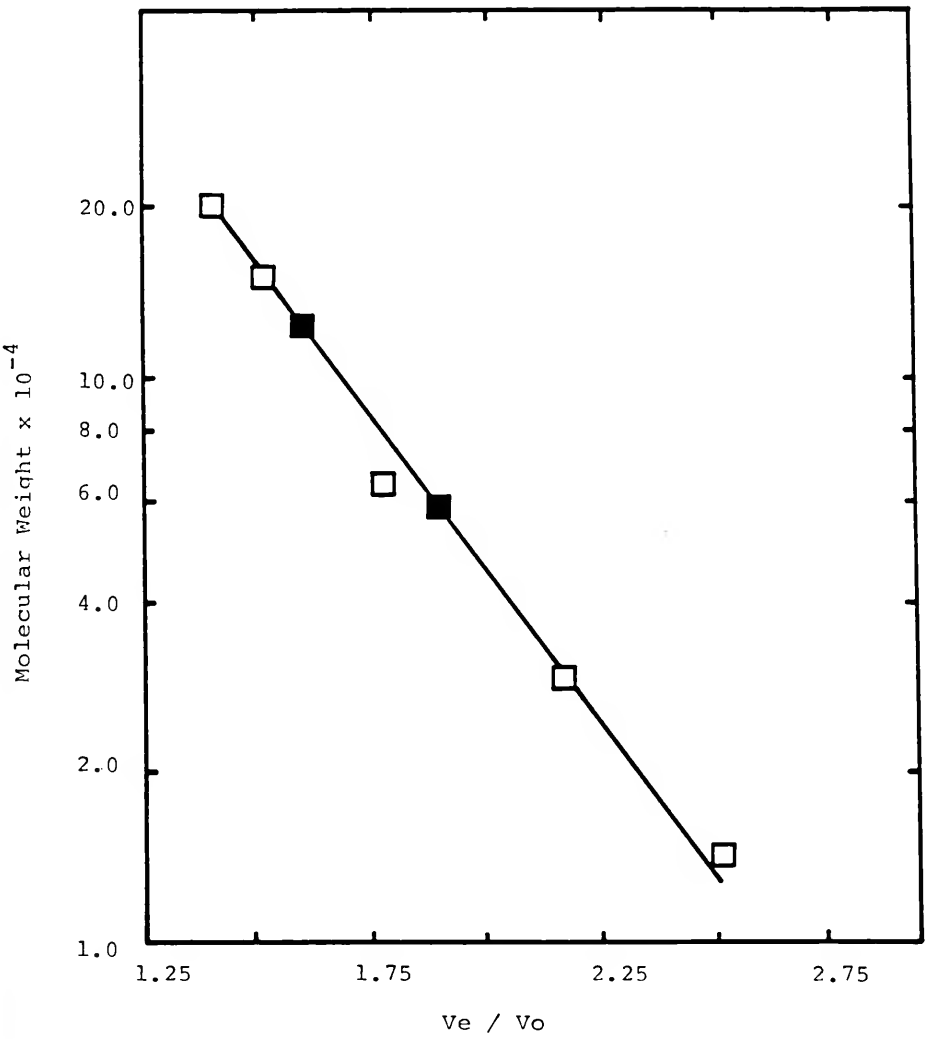
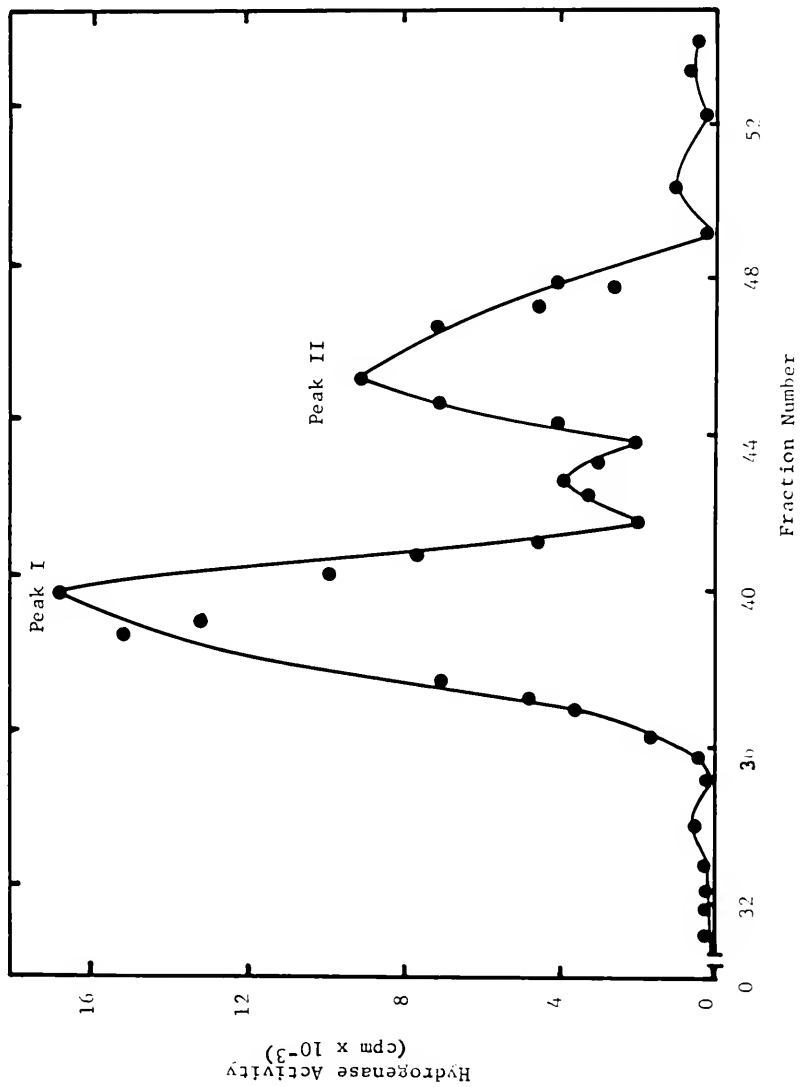

peak II
peak I
Hydrogenase Activity
(cpm x 10 -3)
0
4
8
12
16
0
10
20
30
40
50
60
Fraction Number

Figure 4-4. Calibration curve for the Sephadex G-200 column (2.6 cm x 48 cm) used to determine the molecular weight of hydrogenase in the presence of Triton X-100. □ molecular weight standards; ■ hydrogenase.


Molecular Weight x 10^{-4}
20.0
10.0
8.0
6.0
4.0
2.0
1.0
1.25
1.75
2.25
2.75
Ve / Vo

Gel Filtration in the Absence of Triton X-100: To determine the molecular weight of the enzyme in the absence of Triton X-100, a column of sephadex G-200 (2.6 cm. x 48 cm.)was used and the Ve/Vo values for the molecular weight standard proteins were determined in the same manner as for gel filtration in the presence of Triton X-100 (Table 4-2). The Ve/Vo did not change appreciably for these proteins, in the absence of Triton X-100 except for albumin and carbonic anhydrase which migrated little faster through the column. The Ve/Vo for the enzyme (2.6 microgram/ml; 1.5 ml sample volume) was determined after removing Triton X-100 as described in the Materials and Methods section. The elution profile of hydrogenase was monitored by assaying the different fractions for tritium exchange activity (Figure 4-5). Hydrogenase activity was present in two major peaks. Table 4-2 lists the elution volume (Ve) and the ratio of Ve/Vo for the two hydrogenase peaks and the standard proteins used. The molecular weight of hydrogenase in the two peaks was determined using the caliberation curve (Figure 4-6). Based on three independent determinations, 57% (+/- 9%) of the activity loaded on the column was accounted for in peak I and approximately 33% (+/- 7%) of the activity was detected in peak II. Hydrogenase in peak I had a molecular weight of 125,000 (+/- 10,000) and the hydrogenase in peak II had a molecular weight of 58,000 (+/- 5,000). The minor peak between peak I and peak II had a molecular weight of 77,000 and accounted for 8% of the total activity.

Figure 4-5. Elution profile of hydrogenase in Sephadex G-200 (2.6 cm x 48 cm column), in the absence of Triton X-100.


Peak I
Peak II
Hydrogenase Activity
(cpm x 10-3)
Fraction Number
0
4
8
12
16
32
40
44
48

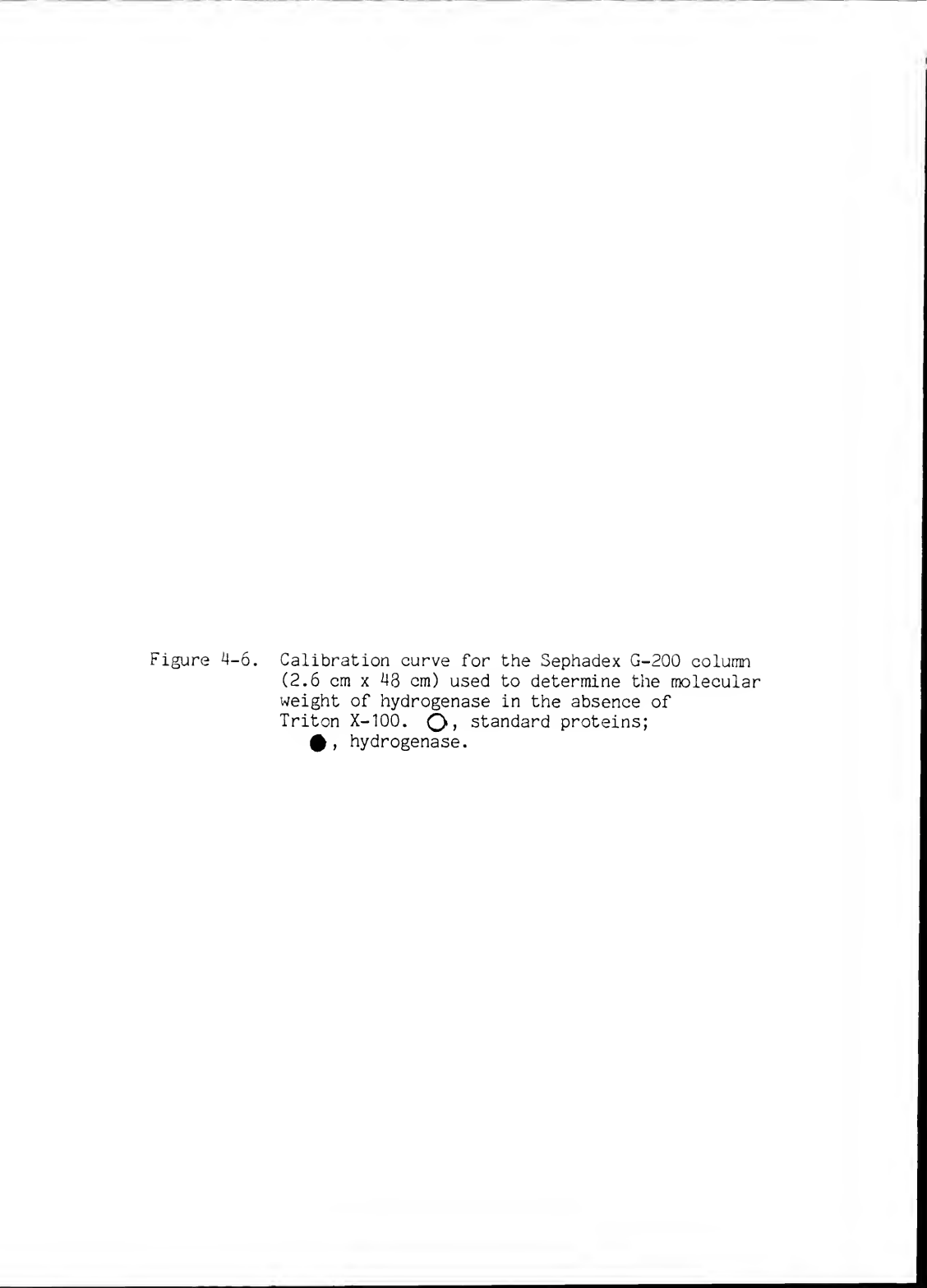
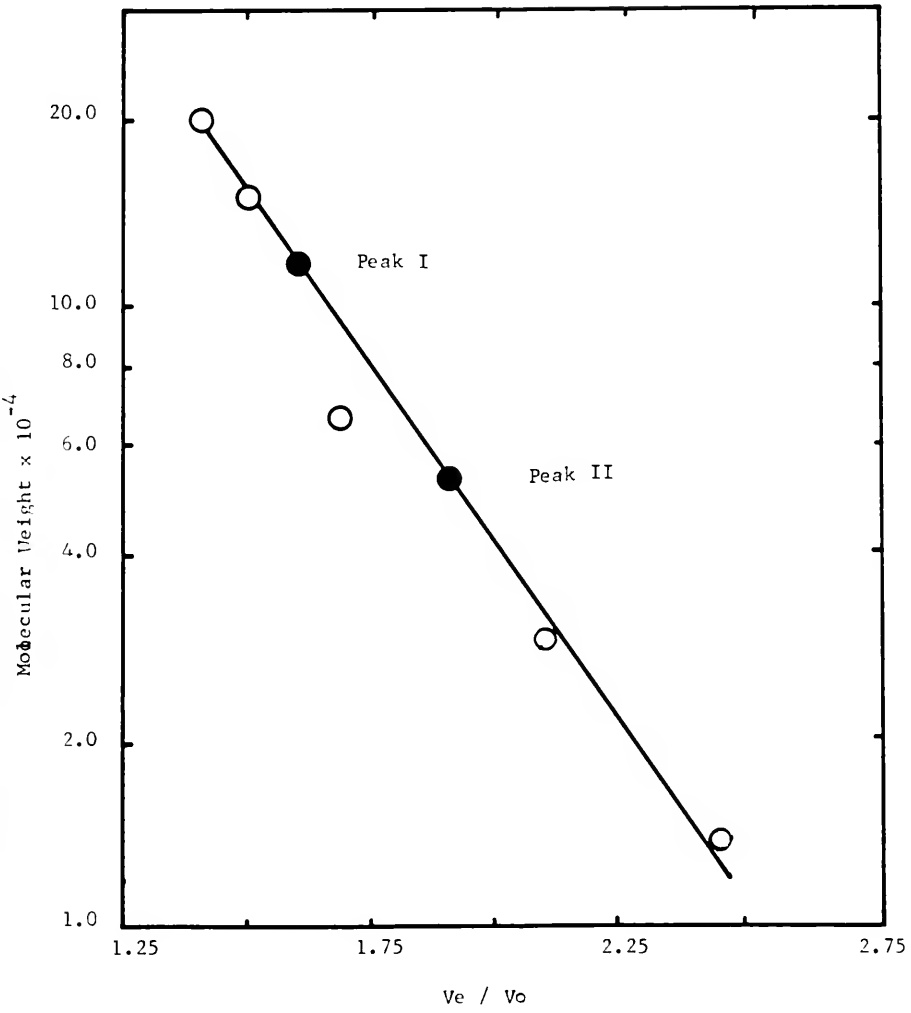


Figure 4-6. Calibration curve for the Sephadex G-200 column (2.6 cm x 48 cm) used to determine the molecular weight of hydrogenase in the absence of Triton X-100. ○, standard proteins; ●, hydrogenase.


20.0
10.0
8.0
6.0
4.0
2.0
1.0
Molecular Weight x 10^{-4}
Peak I
Peak II
1.25
1.75
2.25
2.75
Ve / Vo

Native PAGE In The Presence of Triton X-100: Another independent method used to determine the molecular weight of hydrogenase involved electrophoresis of the enzyme in polyacrylamide gels of different concentrations under non-denaturing conditions. A set of tube gels containing different concentrations of acrylamide was prepared and the samples were subjected to electrophoresis, as described in the Materials and Methods section. The relative mobility of hydrogenase was determined after staining the gels for hydrogenase activity (Figure 4-7, Table 4-3). To determine the molecular weight of hydrogenase, the relative mobilities of proteins with known molecular weights were determined as described in the Materials and Methods section. The Ferguson Plot generated based on the Rf values obtained for the standard proteins is as illustrated in Figure 4-8. The average molecular weight of hydrogenase, based on three independent determinations was 58,000 (+/- 6,000).

Native PAGE in the Absence of Triton X-100: The molecular weight of hydrogenase, using the electrophoresis method was also determined in the absence of Triton X-100. The tube gels were prepared and the samples were subjected to electrophoresis as described in the Materials and Methods section. The only difference in this case was that Triton X-100 was not incorporated in the gels and hydrogenase was free of Triton X-100. The gels were stained for hydrogenase activity after electrophoresis. Under these conditions, two distinct

Figure 4-7. Molecular weight determination of hydrogenase in the presence of Triton X-100 using native PAGE. Polyacrylamide tube gels with different concentrations of acrylamide were loaded with purified hydrogenase (2.6 microgram). Electrophoresis was performed and the gels stained for hydrogenase activity as described in the Materials and Methods section. 1, 5.0% acrylamide; 2, 6.0 % acrylamide; 3, 7.0 % acrylamide; 4, 7.5 % acrylamide; 5, 8.0 % acrylamide and 6, 9.0 % acrylamide.

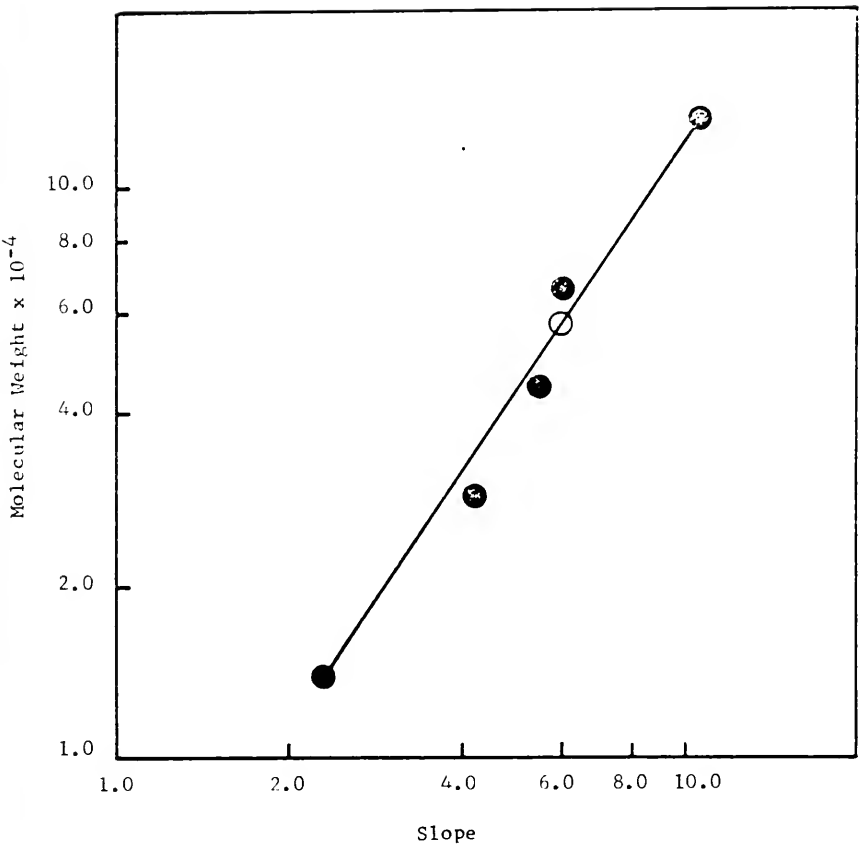

1 2 3 4 5 6

Table 4-3. Relative electrophoretic mobilities (Rf) of hydrogenase and molecular weight standard proteins in the presence of 0.3% Triton X-100, in polyacrylamide gels, at different acrylamide concentrations

Protein	Molecular Weight	Rf at different conc. of acrylamide						
		5%	6%	7%	7.5%	8%	9%	10%
Alpha-Lactalbumin	14,200			0.94		0.84	0.8	0.71
Carbonic Anhydrase	29,000	0.54	0.45	0.39		0.34		
Albumin, Chicken egg	45,000			0.98		0.95	0.83	0.73
Albumin, Bovine Serum (Monomer)	66,000			0.99		0.85	0.78	0.65
Albumin, Bovine Serum (Dimer)	132,000			0.75		0.62	0.48	0.38
Hydrogenase	58,000*	0.94	0.85	0.71	0.63	0.60	0.50	

* Calculated value.

Figure 4-8. Standard curve (Ferguson plot) for the determination of the molecular weight of hydrogenase using native PAGE in the presence of 0.3 % Triton X-100. ○, hydrogenase; ●, molecular weight standard proteins.


Molecular Weight x 10^{-4}
10.0
8.0
6.0
4.0
2.0
1.0
1.0
2.0
4.0
6.0
8.0
10.0
Slope

bands that stained for hydrogenase activity can be observed in the gels (Figure 4-9). The relative migration of both these bands possessing hydrogenase activity and the relative mobilities of the standard proteins are listed in Table 4-4. Figure 4-10 illustrates the Ferguson Plot generated using the Rf values obtained for the molecular weight standard proteins. Based on three independent determinations, hydrogenase with a higher Rf value (band I) corresponds to a molecular weight of 58,000 (+/- 5,000) and the other band (band II) corresponds to a molecular weight of 115,000 (+/- 10,000).

Iron and Sulfur content

The iron and acid labile sulfur content of the enzyme was determined as described in the Materials and Methods section. The two forms of the enzyme were separated by gel filtration. The enzyme with a molecular weight of 125,000 had 8.87 (+/- 0.34) moles of iron and 8.91 (+/- 0.56) moles of sulfur per mole of the enzyme. The 58,000 d enzyme had 4.4 (+/- 0.15) moles of iron and 4.74 (+/- 0.48) moles of sulfur per mole of the enzyme.

Temperature Profile

The optimum temperature for catalysis of the exchange reaction was determined, as described in the Materials and Methods section.

Figure 4-9. Molecular weight determination of hydrogenase in the absence of Triton X-100. Polyacrylamide tube gels with different concentrations of acrylamide were loaded with hydrogenase (2.6 micrograms). After electrophoresis the gels were stained for hydrogenase activity as described in the Materials and Methods section. 1, 4.0 % acrylamide; 2, 6.0 % acrylamide; 3, 7.5 % acrylamide; 4, 10% acrylamide; and lane 5, 12% acrylamide.


1
2
3
4
5

Table 4-4. Relative electrophoretic mobilities (Rf) of hydrogenase and molecular weight standard proteins in the absence of Triton X-100, in polyacrylamide gels, at different acrylamide concentrations.

Protein	Molecular Weight	Rf at different conc. of acrylamide							
		4%	6%	7%	7.5%	8%	9%	10%	12%
Alpha-Lactalbumin	14,200			0.93		0.85	0.30	0.73	
Carbonic anhydrase	29,000		0.46	0.40		0.35			
Albumin, Chicken Egg	45,000			0.98		0.95	0.85	0.74	
Albumin, Bovine Serum (monomer)	66,000			0.99		0.88	0.77	0.66	
Albumin Bovine Serum (Dimer)	132,000			0.76		0.63	0.50	0.40	
Hydrogenase Band I	58,000*	0.98	0.38		0.68			0.42	0.25
Hydrogenase Band II	115,000*		0.76		0.58			0.37	0.23

* Calculated value.

Figure 4-10. Standard curve (Ferguson Plot) for the determination of the molecular weight of hydrogenase using native PAGE in the absence of Triton X-100. , hydrogenase; , molecular weight standard proteins.

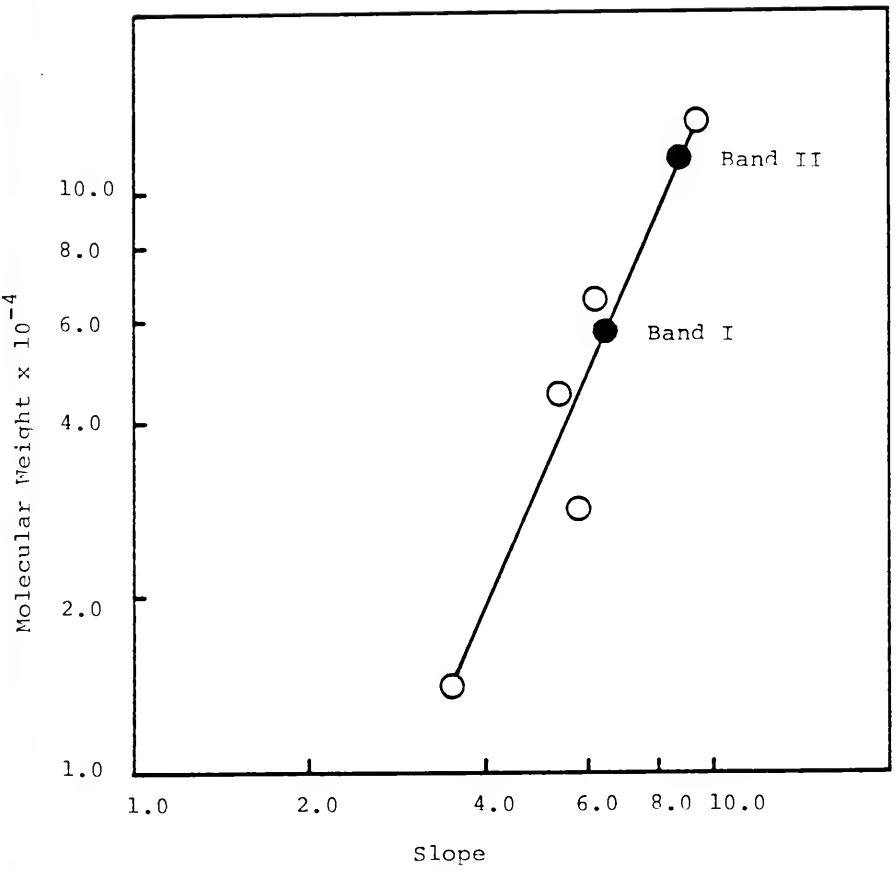
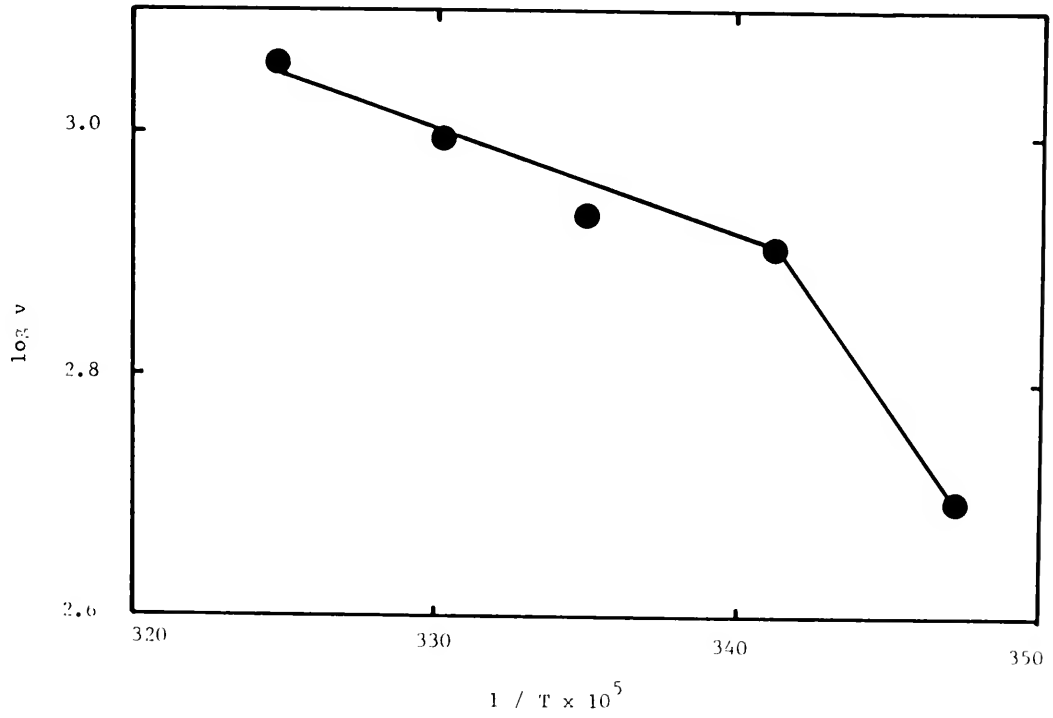

Band II
Band I
10.0
8.0
6.0
4.0
2.0
1.0
Molecular Weight x 10^{-4}
1.0
2.0
4.0
6.0
8.0
10.0
Slope

Figure 4-11 shows the temperature profile for hydrogenase as determined by the exchange reaction. The data represent the results of four independent determinations. As can be deduced from the graph, the optimal temperature for the exchange reaction was 35°C. Figure 4-12 presents an Arrhenius plot for the tritium exchange reaction catalyzed by hydrogenase between 15°C and 35°C. The slope of the line obtained by plotting the log of the reaction rate versus the inverse of the absolute temperature at which the reaction was performed, gives the activation energy for the exchange reaction catalyzed by hydrogenase. Hydrogenase shows two activation energies for the exchange reaction, the activation energy is 867 cal at a temperature range of 35°C to 20°C and 3,517 cal at temperature below 20°C. To determine whether the higher activation energy required at temperatures below 20°C was due to micelle formation by Triton X-100 at lower temperatures, the experiment was also performed in the absence of Triton X-100. The results obtained in the absence of Triton X-100 were similar to the results obtained in the presence of Triton X-100, suggesting that the higher activation energy value observed at temperatures below 20°C was not due to the presence of Triton X-100.

Figure 4-11. Effect of temperature on hydrogenase activity.

Hydrogenase Activity
(micromoles of ^3H_2O produced/mg protein. hour)
1200
800
400
0
10
20
30
40
50
Temperature °C

Figure 4-12. Arrhenius plot for the exchange reaction catalyzed by hydrogenase between the temperature range of 15°C to 35°C.


3.0
2.8
2.6
log v
320
330
340
350
1 / T x 10^5

pH Profile

The optimum pH for the catalysis of the exchange reaction was determined as described in the Materials and Methods section at different pH and in the presence of three different buffers. The buffers used and the pH at which the exchange reaction was carried out were as described in the Materials and Methods section. Figure 4-13 illustrates the pH profile for the exchange activity. A broad pH optimum between pH 7.0 - 7.5 was observed for hydrogenase using the tritium exchange reaction.

Kinetic Characteristics

Since the purified hydrogenase can exist both as a monomer and a dimer, depending on the presence or absence of detergent, it was important to determine whether both the monomer and dimer have the same kinetic properties. To achieve this, the two forms were separated by gel filtration. The apparent Km and Vmax for the exchange reaction were determined for both forms of hydrogenase. The results presented in Table 4-5 show that the apparent Km for hydrogen and the Vmax for the exchange reaction of the monomer and dimer forms of the enzyme are comparable to the apparent Km and the Vmax of the enzyme before the separation. The turn over number calculated based on the molecular weight values obtained using Gel Filtration suggests

Figure 4-13. Effect of pH on hydrogenase exchange activity. (●——●), phosphate buffer; (▲——▲), PIPES buffer and (■——■) Tris HCl buffer. See Materials and Methods section for details.

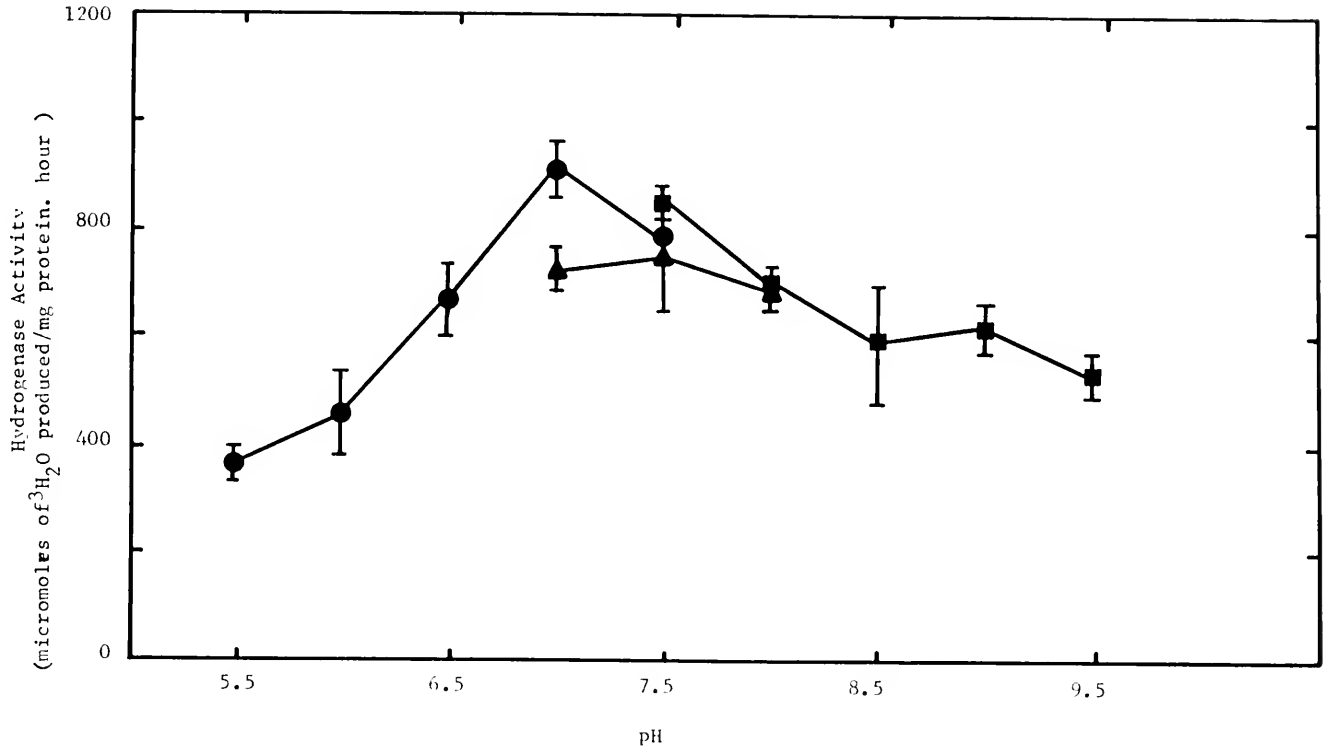

1200
800
400
0
Hydrogenase Activity
(micromoles of ^3H_2O produced/mg protein. hour)
5.5
6.5
7.5
8.5
9.5
pH

Table 4-5. Kinetic Properties of the monomer and the dimer forms of hydrogenase.

Hydrogenase	Tritium Exchange		
	Km[1]	Vmax[2]	Molecular Activity
Before Fractionation	1.6	1780	
Monomer	2.0	1962	107,245
Dimer	2.2	1,780	239,940

[1] micromolar of dissolved hydrogen in the aqueous phase (calculated value based on solubility of hydrogen in solution).

[2] micromoles of $^{3}H_{2}O$ produced/mg. protein. hour.

that the dimer form of the enzyme is composed of two monomers that are equally active.

The apparent Km was also determined for different electron carriers of hydrogenase viz: oxidized benzyl viologen, oxidized methyl viologen, reduced benzyl viologen, reduced methyl viologen and hydrogen. The determinations for each of the substrates were done as described in the Material and Methods section. The computer generated Lineweaver Burk plots for each of the substrates is as presented in Figures 4-14, 4-15, 4-16, and 4-17. The results obtained are an average of three independent determinations and are summarized in Table 4-6. The apparent Km for oxidized methyl viologen in the hydrogen uptake reaction was 26.7 mM and the forward reaction was catalyzed at a maximal velocity of 24.3 micromoles of methyl viologen reduced/min mg protein. Monitoring the rate of hydrogen evolution, the apparent Km for reduced methyl viologen was determined to be 1.5 mM and the Vmax = 35.1 micromoles of hydrogen produced/min. mg protein. The apparent Km for oxidized benzyl viologen in hydrogen uptake reaction was 7.7 mM and the Vmax = 49.4 micromoles of benzyl viologen reduced/min mg protein. The apparent Km for reduced benzyl viologen was 4.0 mM and the maximum velocity at which the rate of hydrogen evolution reaction proceeds, using reduced benzyl viologen as a source of electrons was 12.5 micromoles of hydrogen produced/min. mg protein.

Figure 4-14. Double reciprocal plot (Lineweaver-Burk) of oxidized methyl viologen concentration on hydrogenase activity.

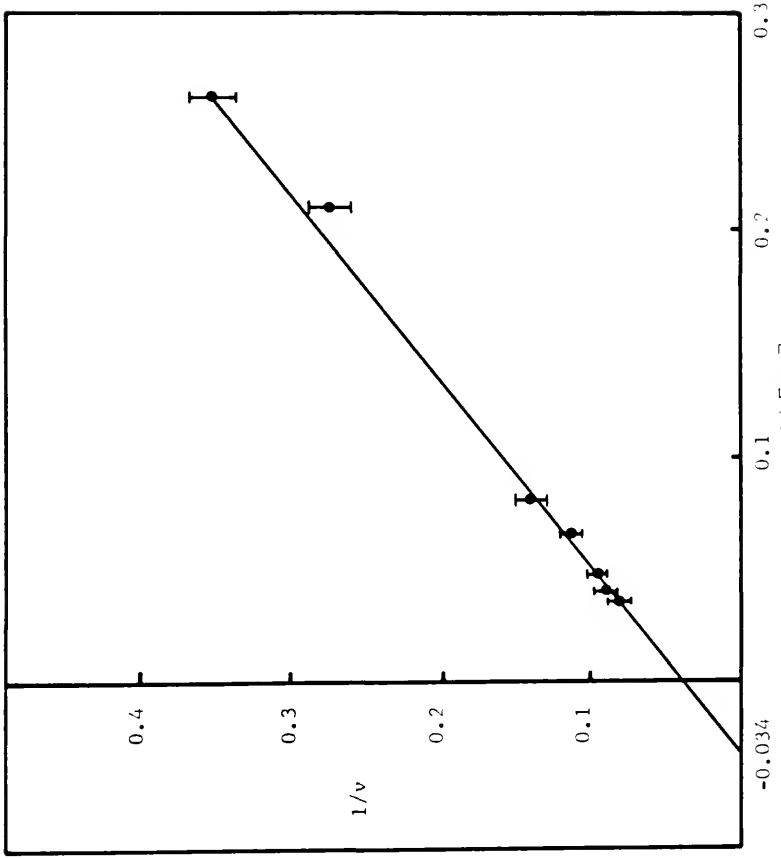

0.4
0.3
0.2
0.1
1/v
-0.034
0.1
0.2
0.3
1/[MV] mM

Figure 4-15. Double reciprocal plot (Lineweaver Burk) of oxidized benzyl viologen concentration on hydrogenase activity.

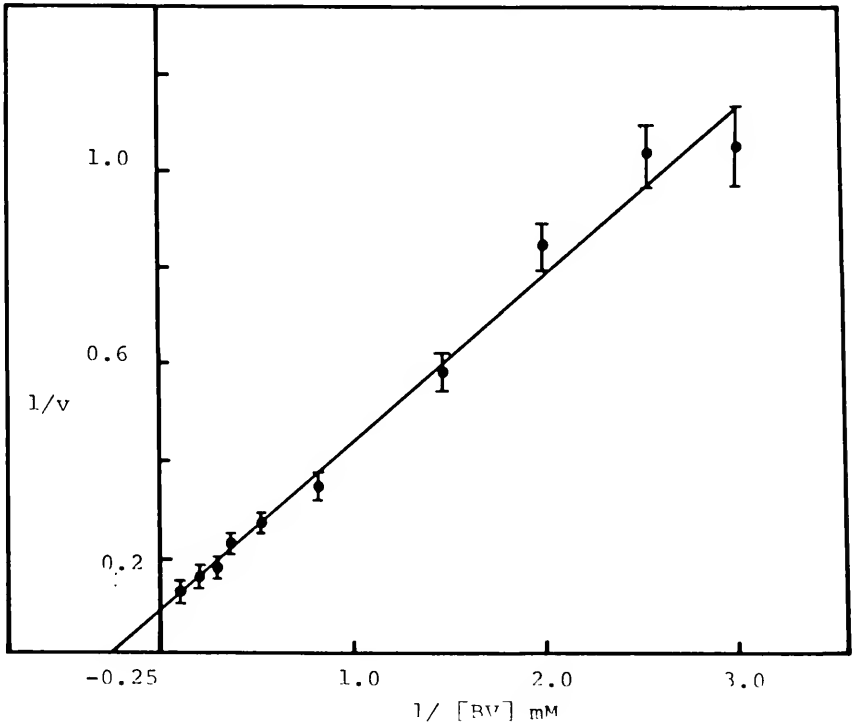

1.0
0.6
1/v
0.2
-0.25
1.0
2.0
3.0
1/ [BV] mM

Figure 4-16. Double reciprocal plot (Lineweaver Burk) of reduced methyl viologen concentration on hydrogenase activity.

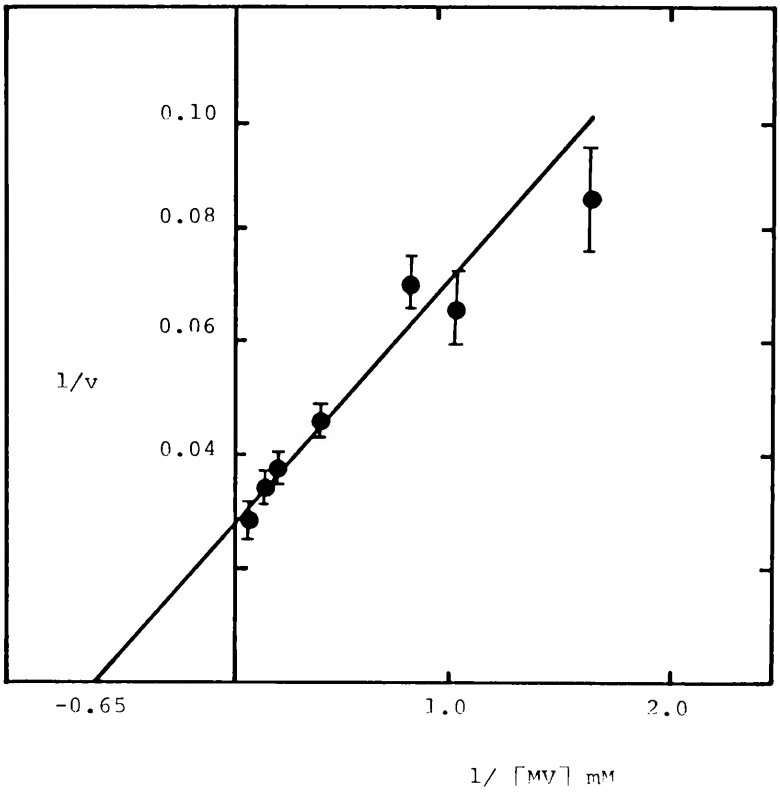

0.10
0.08
0.06
1/v
0.04
-0.65
1.0
2.0
1/ [MV] mM

Figure 4-17. Double reciprocal plot (Lineweaver Burk) of reduced benzyl viologen concentration on hydrogenase activity.

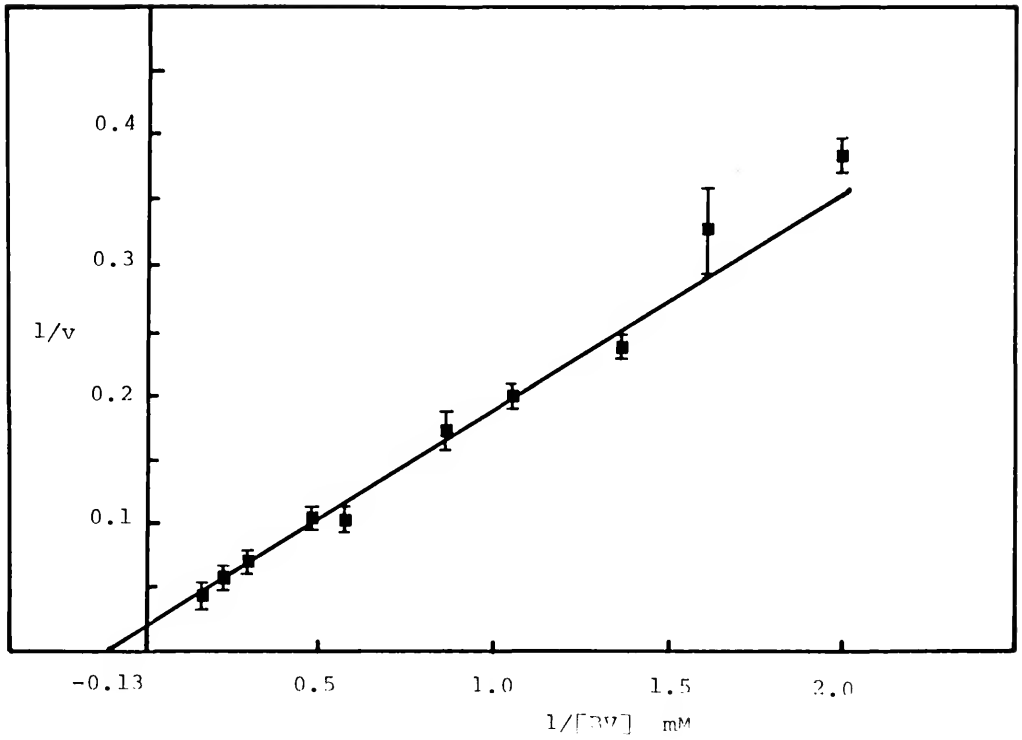

0.4
0.3
1/v
0.2
0.1
-0.13
0.5
1.0
1.5
2.0
1/[BV] mM

Table 4-6. Kinetic Properties of hydrogenase

Substrate	Apparent Km mM	Vmax
Methyl Viologen (oxidized)	26.7	24.3[a]
Benzyl Viologen (oxidized)	7.7	49.4[a]
Methyl Viologen (reduced)	1.5	35.1[b]
Benzyl Viologen (reduced)	4.0	12.5[b]

[a] micromoles of dye reduced/min. mg protein.
[b] micromoles of hydrogen evolved/min. mg protein.

Hydrogen Uptake in the Presence of Different Artificial Electron Acceptors

The ability of hydrogenase to utilize a variety of artificial electron acceptors was also determined. The experiment as described in the Materials and Methods section involved the incubation of hydrogenase with various electron acceptors in the presence of hydrogen and determination of the rate of reduction of the acceptor, spectrophotometrically. Table 4-7 lists the values obtained for the rate of reduction of the various acceptors and their redox potentials (Eo'). As shown in Table 4-7, hydrogenase can use all five compounds as electron acceptors. The results indicate that the rate of hydrogen uptake proceeds at a more rapid rate in the presence of methylene blue and potassium ferricyanide, compounds with a positive Eo'.

Inactivation of Hydrogenase by Oxygen

The inactivation of hydrogenase by oxygen was determined by incubating the enzyme (1.7 microgram/ml) in the presence of air in a 12 x 75 mm tube on a rocking platform at room temperature. At various time intervals aliquots were withdrawn to monitor tritium exchange activity. The control sample of hydrogenase was maintained under similar conditions, but the gas phase was hydrogen. Details of

Table 4-7. Hydrogen uptake activity of hydrogenase in the presence of electron acceptors at different redox potentials.

Electron Acceptor	E_o' (m volts)	Specific Activity *
Methyl Viologen	-440	22.3
Benzyl Viologen	-360	33.2
Neutral red	-325	18.4
Methylene Blue	+ 11	157.0
Potassium ferricyanide	+360	193.0

* micromoles of substrate reduced/min. mg protein.

the experiment are as described in the Materials and Methods section. Figure 4-18 gives the oxygen inactivation profile of the hydrogenase exposed to air and the hydrogenase sample maintained under hydrogen. The half-life of the hydrogenase in the presence of air was 650 minutes. The inactivation of the monomer and the dimer form of hydrogenase due to oxygen was also determined. The two forms of hydrogenase were obtained by Gel Filtration as described in the Materials and Methods section. The experiment was performed as described abvove. The inactivation profile for the first thirty minutes is as illustrated in Figure 4-19. It is apparent that both the monomer and dimer form of hydrogenase are equally stable in air.

Stability of Hydrogenase at Alkaline pH

Ballantine and Boxer (10) recently reported the presence of two different hydrogenases in E. coli. They detected two distinct bands of hydrogenase activity when Triton X-100 solubilized membranes were subjected to PAGE at neutral pH. They observed that one of these bands was labile at alkaline pH (pH 10.0), and lost the hydrogenase activity. Thus the stability of the purified hydrogenase at alkaline pH was checked. Hydrogenase was incubated at pH 10.0 for 15 minutes and then the activity was determined by monitoring the exchange reaction and the hydrogen uptake reaction at, pH 7.0 and pH 10.0. For the exchange reaction, 75 microliters of 100 mM glycine, pH 10.0,

or 100 mM phosphate buffer, pH 7.0, was placed in a 12 x 75 mm tube. The tubes were capped with serum stoppers and the gas phase replaced with helium. Hydrogenase (25 microliters, 12.0 microgram/ml in 10 mM phosphate buffer, pH 7.0) was added to the tube and incubated at room temperature for 15 minutes. After 15 minutes, the pH of the assay mixture was brought to neutrality or maintained at pH 10.0, by adding 100 microliters of 100 mM phosphate buffer, pH 6.5, or 100 microliters of 100 mM glycine buffer, pH 10.0. Tritium exchange reaction was performed as described in the Materials and Methods section. For the hydrogen uptake reaction, 500 microliters of either 100 mM glycine buffer, pH 10.0, or phosphate buffer, pH 7.0, was placed in a 12 x 75 mm tube. The tubes were capped with serum stoppers and the gas phase replaced with hydrogen. Hydrogenase (100 microliters, 12.0 microgram/ml in 10 mM phosphate buffer, pH 7.0) was added and incubated at room temperature for 15 minutes. After 15 minutes, the pH of the assay mixture was brought to neutrality or maintained at pH 10.0 by adding 1.9 ml of either phosphate buffer, pH 7.0, or 100 mM glycine buffer, pH 10.0. The rate of hydrogen uptake reaction was determined by monitoring the reduction of benzyl viologen as described in the Materials and Methods section. The results presented in Table 4-8 show that the enzyme retained 62.5% of its exchange activity and 76.5% of its hydrogen uptake activity after incubation at pH 10.0 for 15 minutes. It is also evident that the

Figure 4-18. Oxygen inactivation of hydrogenase. (●—— ●), control; (● ●) hydrogenase exposed to oxygen.

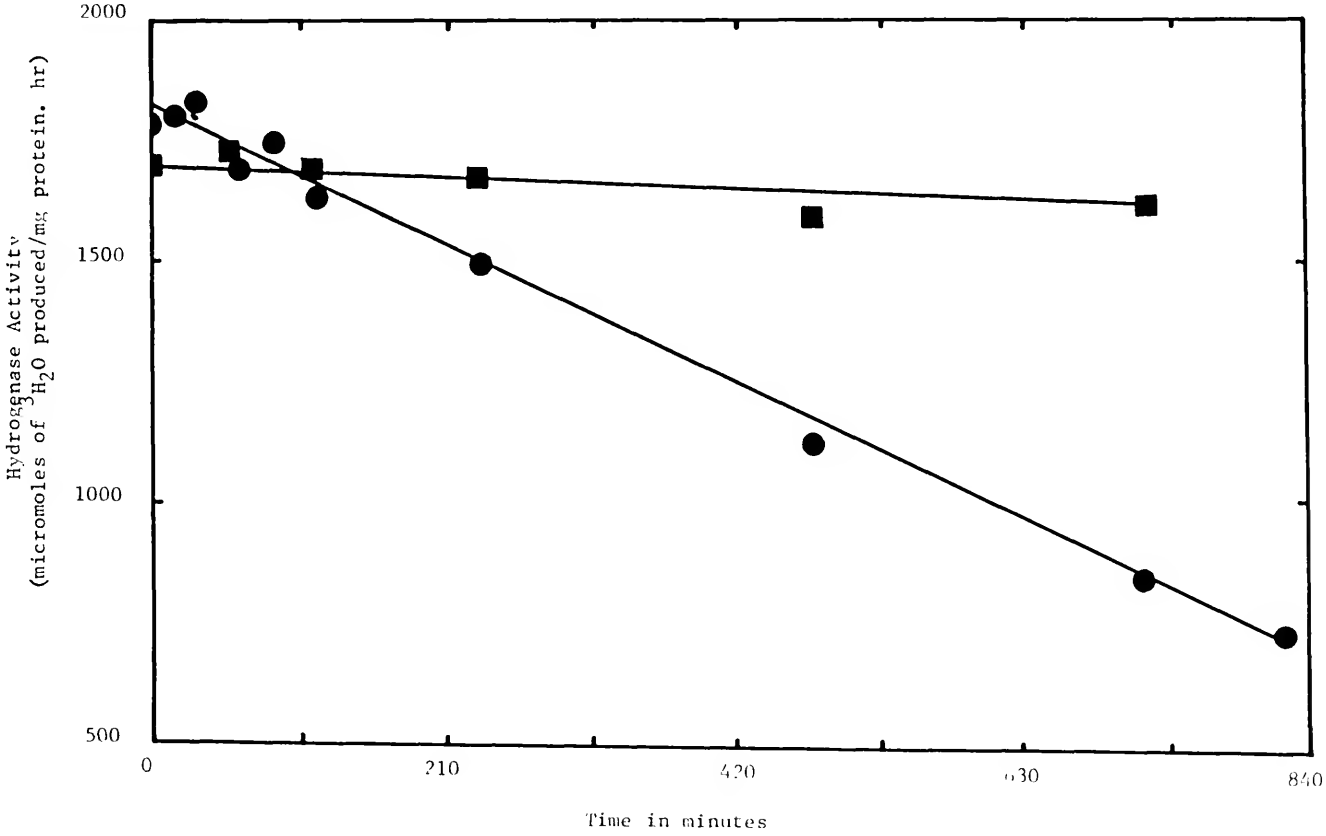

2000
1500
1000
500
0
210
420
630
840
Hydrogenase Activity
(micromoles of ^3H_2O produced/mg protein. hr)
Time in minutes

Figure 4-19. Oxygen inactivation of the monomer and dimer forms of hydrogenase
A, monomer; B, dimer.

Hydrogenase activity
(micromoles of ^3H_2O produced/mg. hour)
% of control
100
90
80
A
B
0
8
16
24
32
Time in minutes

Table 4-8. Stability of hydrogenase at alkaline pH.

Pre-incubation pH	Assay pH	Hydrogenase Activity*	
		Exchange[1]	HUP[2]
7.0	7.0	1586	37.4
10.0	7.0	989	28.6
10.0	10.0	365	8.3

[1] micromoles of $^{3}H_{2}O$ produced/hour mg protein.

[2] micromoles of BV reduced/mg. protein. min.

enzyme is capable of catalyzing the exchange reaction and the hydrogen uptake reaction at pH 10.0 (22% of the pH 7.0 value).

Hydrogenase Activity in Solubilized Membranes of HUP⁻ Mutants of Escherichia coli.

To determine the possibility of two distinct hydrogenases being present in E. coli, one involved in the FHL activity and the other involved in the HUP activity, mutants defective in the HUP activity were studied. For these experiments E. coli strains lacking HUP activity but possessing normal levels of FHL activity were obtained (55). Lambden and Gest have suggested that the fnr gene product may be involved in the regulation of hydrogenase activity and this was confirmed in our laboratory. Thus, an fnr mutant strain of E. coli was also studied. Triton X-100 solubilized membranes of E. coli strains JC-10244 and selected HUP⁻ mutants, SE-8, SE-49 and an fnr mutant, SD-7 were prepared as described earlier. Solubilized membrane samples of these strains and pure hydrogenase protein (0.084 units of hydrogenase activity, as determined by the tritium exchange reaction) were subjected to electrophoresis in 7.5% acrylamide in tube gels under non-denaturing conditions. The tube gels, after electrophoresis were stained for hydrogenase activity (Figure 4-20). E. coli strain JC-10244, wild type for hydrogen metabolism, had two hydrogenase activity bands with Rf values of 0.15 and 0.6. The

Figure 4-20. Hydrogenase activity of Triton X-100 solubilized membranes of E. coli HUP^+ and HUP^- strains and purified hydrogenase subjected to native-PAGE in tube gels.
Triton X-100 solubilized membranes of E. coli strains JC-10244, SE-8, SE-49 and SD-7 were subjected to native PAGE and stained for hydrogenase activity as described in the text. A, JC-10244; B, SE-8; C, SE-49; D, SD-7 and E, hydrogenase.

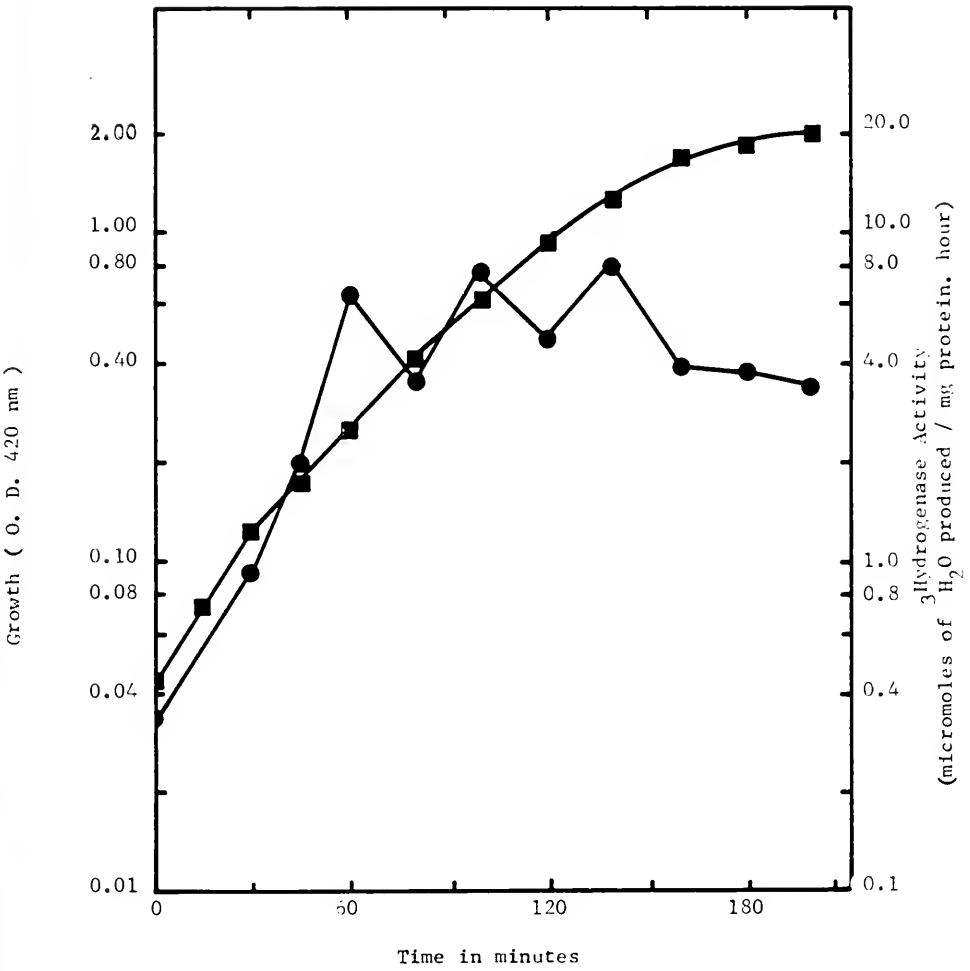
A B C D E

purified hydrogenase migrated with an Rf of 0.63. On the other hand, HUP^- strains SE-8, SE-49, and SD-7 produced only a very faint band of hydrogenase activity with an Rf value of 0.15.

Regulation of Hydrogenase Activity in Whole Cells

Induction of Hydrogenase Activity: E. coli cells, grown under strict aerobic conditions lack hydrogenase activity. However, as soon as the culture is depleted of oxygen, hydrogenase activity is induced. This induction of hydrogenase activity was studied by shifting an actively growing aerobic culture to growth under anaerobic conditions. The experiment, as described in the Materials and Methods section was performed by shifting an aerobically growing culture of E. coli strain K-10 to growth under anaerobic conditions by incubating the culture under an atmosphere of nitrogen. Samples were withdrawn at twenty minutes interval to monitor growth and hydrogenase activity. As shown in Figure 4-21 the culture had a generation time of 39 minutes. The hydrogenase activity, monitored as tritium exchange activity, was induced to a maximum level within the first hour after shifting the culture to anaerobic conditions. This maximum specific activity of approximately 7.0 units then stabilized to a lower value of 3.0 units. This activity is maintained throughout the rest of the growth period.

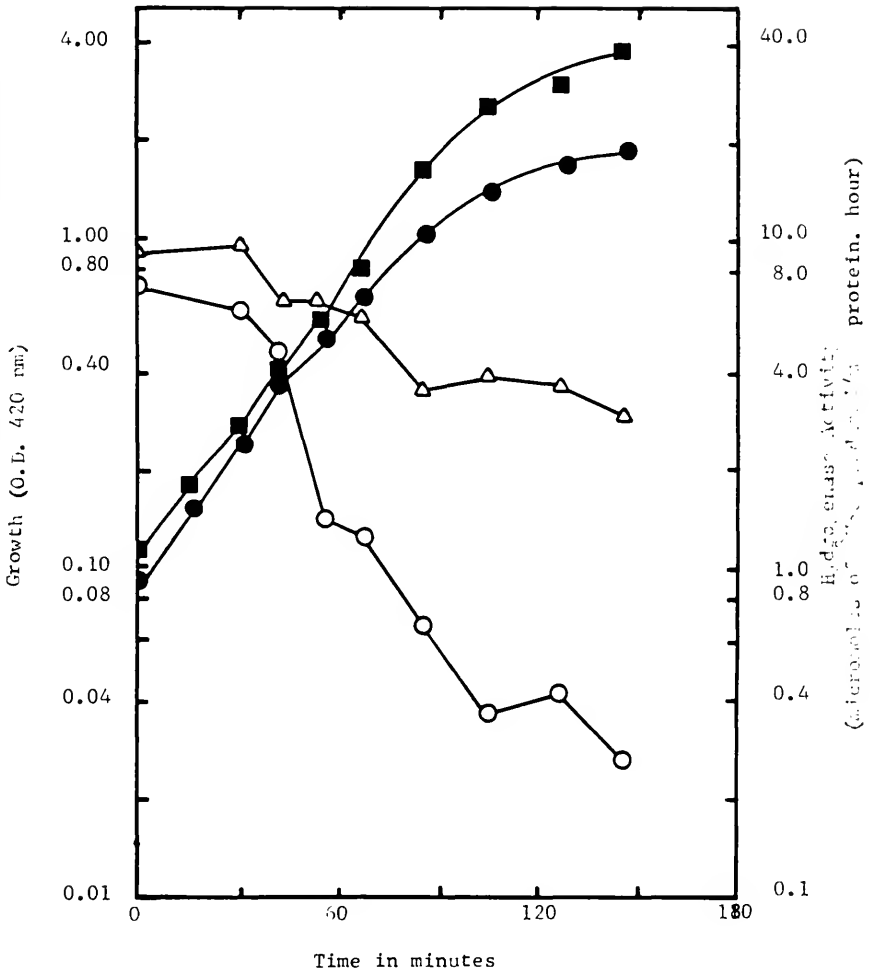
Figure 4-21. Induction of hydrogenase activity in E. coli. Growth of the culture (■———■); Hydrogenase activity (●———●).


2.00
1.00
0.80
0.40
0.10
0.08
0.04
0.01
0
60
120
180
Time in minutes
Growth (O. D. 420 nm)
20.0
10.0
8.0
4.0
1.0
0.8
0.4
0.1
^{3}Hydrogenase Activity
(micromoles of H_2O produced / mg protein. hour)

Effect of Oxygen on Hydrogenase Activity in Whole Cells: Hydrogenase is inactivated by oxygen, and a culture growing aerobically lacks hydrogenase activity. Thus it was interesting to determine the effect oxygen would have on a growing culture of E. coli fully induced for hydrogenase activity and study the kinetics of the repression of hydrogenase activity. The experiment was performed as described in Materials and Methods section. Figure 4-22 presents the growth profile and the hydrogenase activity of the two cultures. As can be seen in the figure, the hydrogenase activity in the culture exposed to oxygen decreased from a specific activity of 7.0 units to 1.0 unit in the first twenty minutes and then declined further to a specific activity of 0.4 units in the next twenty minutes, whereas the culture kept under anaerobic conditions maintained its hydrogenase activity at a steady state level of 3.0 units. The half life of the hydrogenase activity in the cells exposed to oxygen was 8 minutes.

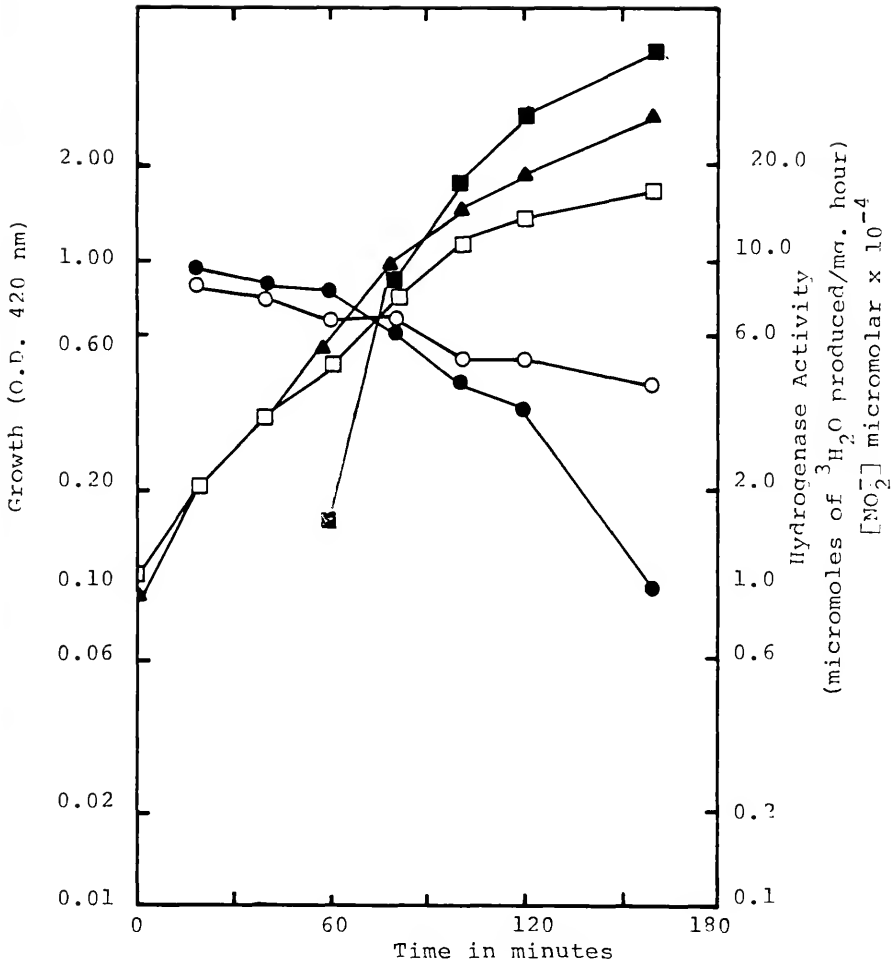
Regulation of Hydrogenase Activity in the Presence of Nitrate: A culture of E. coli when grown in the presence of nitrate does not produce hydrogenase activity or evolve hydrogen gas. To understand this effect of nitrate on hydrogenase activity, nitrate was added to a fully induced culture and the growth profile and hydrogenase activity of the culture were studied as described in the Materials

Figure 4-22. Effect of Oxygen on hydrogenase activity in E. coli.
Growth of the culture exposed to air (▪ - - -▪); Growth of the control culture, (●——●); Hydrogenase activity of culture exposed to air, (△———△); Hydrogenase activity of control culture, (○ ——— ○).


4.00
1.00
0.80
0.40
0.10
0.08
0.04
0.01
40.0
10.0
8.0
4.0
1.0
0.8
0.4
0.1
0
60
120
180
Time in minutes
Growth (O.D. 420 nm)
protein. hour)

and Methods section (Figure 4-23). It is interesting to note that the loss of hydrogenase activity in the culture to which nitrate was added was bi-phasic. Hydrogenase activity declined gradually immediately after the addition of nitratewith a half life of about 40 minutes. However, after 80 minutes the hydrogenase activity of the nitrate culture dropped rapidly with a half life of 10 minutes. It is possible the initial gradual decline in hydrogenase activity is due to the inhibition of synthesis of any new hydrogenase after the addition of nitrate and the dilution of the hydrogenase already synthesized due to growth. The more rapid decline in hydrogenase activity may be a result of inhibition of hydrogenase activity by sodium nitrite, a product of nitrate respiration, which accumulated in the medium.

Figure 4-23. Effect of sodium nitrate on hydrogenase activity in whole cells of E. coli K-10. Sodium nitrate (11.7 6mM) was added at 40 minutes. Growth of the control culture, (●——●); Growth of the culture in the presence of sodium nitrate, (▲——▲); Hydrogenase activity of the control culture, (○—— ○); Hydrogenase activity of the culture with sodium nitrate, (▷— ▷); Concentration of nitrite in the medium (▽—— ▽).


Growth (O.D. 420 nm)
2.00
1.00
0.60
0.20
0.10
0.06
0.02
0.01
0
60
120
180
Time in minutes
20.0
10.0
6.0
2.0
1.0
0.6
0.2
0.1
Hydrogenase Activity
(micromoles of ^3H_2O produced/mg. hour)
$[NO_2^-]$ micromolar x 10^{-4}

DISCUSSION

Escherichia coli, under fermentative conditions evolves hydrogen from formate via the formate hydrogenlyase enzyme complex and hydrogenase is an integral part of this complex (5). E. coli also has the ability to utilize hydrogen as a source of reducing power (5). Again, the enzyme hydrogenase coupled with other electron carriers and fumarate reductase, is involved in the hydrogen uptake (69). However, as mentioned in the Literature Review section, it is not known whether the same hydrogenase is involved in both these reactions or whether there are two distinct hydrogenases, one of which is a part of the formate hydrogenlyase complex and the other responsible for the uptake of hydrogen. The presence of two distinct hydrogenases in the same cell has been demonstrated in a number of microorganisms (54,67,74). Ballantine and Boxer (11) have recently suggested the presence of two hydrogenases in E. coli also, but the evidence presented by these authors is indirect and the question of the number of hydrogenases is still open.

The hydrogenase purified from E. coli and characterized as described in the Results section exists predominantly as a monomer in the presence of Triton X-100. Removal of the detergent shifted the ratio of monomer to dimer in favor of the dimer. This observation

may explain the conflicting reports with respect to the molecular weight of hydrogenase from E. coli. Adams and Hall (4) reported a molecular weight of 113,000 as determined by gel filtration. Based on SDS-Page experiments they concluded that the enzyme exists as a dimer with subunits of the same molecular weight. These investigators did not attempt to separate and assay the monomer for activity. Graham (35) reported a molecular weight of 63,000 for hydrogenase immunoprecipitated from Triton X-100 solubilized E. coli membranes. The detergent used by Adams and Hall (4) to solubilize the hydrogenase from the membrane was sodium deoxycholate (1% w/v), and was used early in the purification procedure. The detergent was not incorporated in any of the buffers used during the later stages of purification. The lack of detergent in the buffers could have resulted in the complete removal of the detergent or its concentration reduced to such low levels that the enzyme existed only as a dimer with a resultant molecular weight of 113,000. In light of the results cited in the previous section, it is evident that the hydrogenase from E. coli exists as a monomer in the presence of a detergent and as a dimer in its absence. Sim and Sim (95) reported similar properties for a hydrogenase purified from P. denitrificans. This hydrogenase was found to exist as a dimer in the presence of a detergent and as a tetramer in the absence of a detergent. Since the detergent mainly disrupts hydrophobic forces and the purified hydrogenase from E. coli aggregates to form only a dimer and not

multimers, it is likely that the enzyme exists as a dimer in the native state in E. coli. If the enzyme does exist as a dimer, results in Table 4-5 show that the dimer is made up of two subunits that have the same kinetic properties as determined using the exchange reaction and have the same iron and acid labile sulfur (4 atoms per molecule of subunit). However, more experiments need to be performed to demonstrate that the two subunits are identical.

The purified enzyme is capable of catalyzing both the hydrogen uptake as well as the hydrogen evolution reaction in the presence of appropriate electron donors or acceptors. The pH optimum for the catalysis of the exchange reaction lies in the physiological range of 7.0 and 7.5, and the temperature optimum is 35°C. It is interesting to note that the activation energy for the exchange reaction catalyzed by hydrogenase is 875 calories between the temperature range of 35° C to 20°C. The activation energy is increased to 3,517 calories for the reaction at temperatures below 20°C. The comparatively low activation energy observed may be a consequence of the reaction being monitored in these studies, is an exchange reaction. The purified enzyme is relatively stable in the presence of air. It has a half life of about 650 minutes at room temperature. Adams and Hall (4) reported a half life of 720 minutes in the presence of air for the hydrogenase they purified.

The physiological role of the purified hydrogenase has not been established conclusively. However, kinetic characteristics of the

purified protein (Table 4-6) indicate that the enzyme is probably involved in the hydrogen uptake activity and not in the hydrogen evolution. This apparent role is further confirmed by the results obtained with E. coli mutants deficient in the hydrogen uptake activity. Two different types of HUP^- mutants, Class I mutants as described by Lee et al.(65) and a regulatory mutant (fnr) did not produce any hydrogenase activity that corresponded to the hydrogenase purified from the wild type after native PAGE.

Another indication that the purified hydrogenase is the enzyme involved in the hydrogen uptake reaction in E. coli is the stability of the enzyme in the presence of oxygen. The HUP hydrogenases from chemo-lithotrophs, is known to couple hydrogen oxidation, through other electron carriers, to the reduction of electron acceptors such as sulfate, nitrate and even oxygen. Thus from an evolutionary point of view the HUP hydrogenase would be expected to be more resistant to inactivation by oxygen as compared to the hydrogenase involved in the FHL reaction. This is reflected in the survey of the literature (5). The oxygen sensitivity of hydrogenase appears to correlate with the physiological function the enzyme performs rather than with the oxygen sensitivity of the organism from which the enzyme is derived. The hydrogenase involved in the production of hydrogen is more sensitive to inactivation by oxygen compared to the hydrogenase involved in the hydrogen uptake reaction.

It is interesting to note that the rate at which the enzyme catalyzes the evolution of hydrogen from reduced benzyl viologen is considerably lower than the rate at which it catalyzes the hydrogen uptake reaction in the presence of oxidized benzyl viologen. This observation is similar to the results obtained by Chen et al. (17) for the hydrogen uptake hydrogenase from Clostridium pasteurianum and by Van der Werf and Yates (108) for the uptake hydrogenase from Azotobacter and by Arp et al. (8) for the HUP hydrogenase from Rhizobium. Thus it is possible that the purified hydrogenase in this study is the enzyme involved in the hydrogen uptake reaction in E. coli. However, more experiments need to be performed to establish this fact. Evidence is slowly accumulating with respect to the regulation of hydrogenase in E. coli. The enzyme is inducible and is induced only under anaerobic conditions. Reviewing Figure 4-21, it is apparent that the enzyme activity is detected within sixty minutes after the culture is shifted to growth under anaerobic conditions. The maximal levels of activity is detected in the mid-exponential phase of the growth cycle. This activity declines slightly and stabilizes to a specific activity of 3 units during the late exponential and stationery phase of the culture.

The reverse of induction is observed when a culture fully induced for hydrogenase is shifted to growth under aerobic conditions. Hydrogenase activity declined very rapidly and dropped to below 5 % of the maximally induced levels within sixty minutes.

It is interesting to note that though the purified enzyme is relatively stable in the presence of air, the hydrogenase activity in whole cells drops far more rapidly upon exposure to air (half life of 8 minutes) than what can be accounted for by oxygen inactivation alone. It is possible that the cell degrades the hydrogenase during the rearrangement of the membrane upon exposure to air.

The effect of sodium nitrate, another compound that can be used as a terminal electron acceptor for anaerobic respiration, however, is not so drastic. Cultures growing in the presence of 11.76 mM sodium nitrate lack hydrogenase activity. However, if the same concentration of sodium nitrate is added to an actively growing culture maximally induced for hydrogenase, the hydrogenase specific activity of the culture declined with a bi-phasic kinetics. The initial gradual decline can be easily attributed to lack of any new synthesis of hydrogenase and dilution of the already synthesized hydrogenase due to growth. The more rapid decline with a half life of 10 minutes may be due to inhibition of hydrogenase activity due to the accumulation of sodium nitrite. This half life of 10 min. is similar to the $t_{1/2}$ life of 8 min. in the presence of oxygen.

It should be stated here that the purification procedure presented for the purification of a membrane bound hydrogenase from E. coli does not involve the use of any proteases. A number of researchers have resorted to the use of denaturing detergents or proteolytic agents or a combination of both to achieve solubilization

of a membrane bound protein. However, it is possible that the use of such reagents may yield a protein which does not reflect the true properties of the native protein.

In conclusion, this report, like any other scientific report should, answers a few questions and in the process asks new questions. The purification of hydrogenase should facilitate the production of antibodies directed against the enzyme. The antibodies can be used to elucidate the juxtaposition of the enzyme in the membrane and to study the regulation of synthesis of the protein. Experiments performed with the antibodies should help us determine the number of hydrogenases in the cell and their physiological function. With the pure protein, experiments can also be performed to test the feasibility of the enzyme as a catalyst in cell free systems to produce hydrogen as a fuel source (13,86,87).

REFERENCES

1) Ackrell, B.A.C., R.N. Asato, and H.F. Mower. 1966. Multiple Forms of Bacterial Hydrogenases. J. Bacteriol. 92:828-838.

2) Adams, M.W.W., and D.O. Hall. 1977. Isolation of the Membrane Bound Hydrogenase from Rhodospirillum rubrum. Biochem. Biophys. Res. Comm. 77:730-737.

3) Adams, M.W.W., and D.O. Hall. 1979. Properties of the Membrane-Bound Hydrogenase of the Photosynthetic Bacterium Rhodospirillum rubrum. Arch. of Biochem. Biophys. 195:288-299.

4) Adams, M.W.W., and D.O. Hall. 1979. Purification of the Membrane Bound Hydrogenase of Escherichia coli. Biochem. J. 183:11-22.

5) Adams, M.W.W., L.E. Mortenson, and J.S. Chen. 1981. Hydrogenase. Biochim. et Biophys. Acta 594:105-176.

6) Aketagawa, J., K. Kobayashi, and M. Ishimoto. 1983. Characterization of Periplasmic Hydrogenase from Desulfovibrio vulgaris Miyazaki K. J. Biochem. 93:755-762.

7) Albracht, S.P.J., K.J. Albrecht-Ellmer, D.J.M. Schmedding, and E.C. Slater. 1982. On the Active Site of Hydrogenase from Chromatium vinosum. Biochim. et Biophys. Acta 681: 330-334.

8) Arp, D.J. 1985. Rhizobium japonicum Hydrogenase: Purification to Homogeneity from Soybean Nodules and Molecular Characterization. Arch. of Biochem. Biophys. 237:504-512.

9) Bachmann, B.J. 1983. Linkage Map of Escherichia coli K-12, Edition 7. Microbiol. Rev. 47:180-230.

10) Balch, W.E., G.E. Fox, L.J. Magrum, C.R. Woese, and R.S. Wolfe. 1979. Methanogens: Reevaluation of a Unique Biological Group. Microbial. Rev. 43:260-296.

11) Ballantine, S.P., and D.H. Boxer. 1985. Nickel Containing Hydrogenase Isoenzymes from Anaerobically Grown Escherichia coli K-12. J. Bacteriol. 163:454-459.

12) Bell, G.R., J.P. Lee, and H.G. Peck, Jr. 1978. Reactivity of Desulfovibrio gigas Hydrogenase toward Artificial and Natural Electron Donors or Acceptors. Biochimie. 60:315-320.

13) Berenson, J.A., and J.R. Benemann. 1977. Immobilization of Hydrogenase and Ferredoxin on Glass Beads. FEBS Lett. 76: 105-107.

14) Bernhard, T.H., and G. Gottschalk. 1978. The Hydrogenase of Escherichia coli, Purification, Some Properties and the Function of the Enzyme. In Hydrogenases: Their Catalytic Activity, Structure and Function, ed. Schlegel, H.G., and K. Schneider. E. Goltze KG, Gottingen. 151-158.

15) Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72:248-254.

16) Chen, J.S., and D.K. Blanchard. 1978. Isolation and Properties of a Unidirectional H_2-Oxidizing Hydrogenase from the Strictly Anaerobic N-Fixing Bacterium Clostridium pasteurianum W5. Biochem. Biophys. Res. Comm. 84:1144-1150.

17) Chen, J.S., and L.E. Mortenson. 1974. Purification and Properties of Hydrogenase from Clostridium pasteurianum W5. Biochim. et Biophys. Acta 364-283-298.

18) Colbeau, A., J. Chabert, and P.M. Vignais. 1978. Hydrogenase Activity in Rhodopseudomonas capsulata Stability and Stabilization of the Stabilized Enzyme. In Hydrogenases: Their Catalytic Activity, Structure and Function, ed. Schlegel, H.G., and K. Schneider. E. Goltze KG, Gottingen. 183-197.

19) Colbeau, A., J. Chabert, and P.M. Vignais. 1983. Purification, Molecular Properties and Localization in the Membrane of the Hydrogenase of Rhodopseudomonas capsulata. Biochim. et Biophys. Acta 748:116-127.

20) Colbeau, A., and P.M. Vignais. 1983. The Membrane-Bound Hydrogenase of Rhodopseudomonas capsulata Is Inducible and Contains Nickel. Biochim. et Biophys. Acta 748:128-138.

21) Davis, B.J. 1964. Disc Electrophoresis-II: Method and Application to Human Serum Proteins. Annal. N.Y. Acad. Sci. 121_ 404-427.

22) Egerer, P., H. Gunther, and H. Simon. 1982. On the Hydrogen-Deuterium Exchange Reaction Catalyzed by the Soluble Hydrogenase from Alcaligenes eutrophus H16 in the Free and Immobilized State. Biochim. et Biophys. Acta 703:149-157.

23) Egerer, P., and H. Simon. 1982. Isotropic and Kinetic Studies and Influence of Dicoumarol on the Soluble Hydrogenase from Alcaligenes eutrophus H16. Biochim. et Biophys. Acta 703: 158-170.

24) Farkas, A., L. Farkas, and J. Yudkin. 1934. The Decomposition of Sodium Formate by Bacterium coli in the Presence of Heavy Water. Proc. Royal Soc. of Lon. B 115:373-378.

25) Ferguson, K.A. 1964. Starch Gel Electrophoresis--Application to the Classification of Pituitary Proteins and Polypeptides. Metabolism 13:985-1002.

26) Gest, H. 1952. Properties of Cell-Free Hydrogenases of Escherichia coli and Rhodospirillum rubrum. J. Bacteriol. 63:111-121.

27) Gest, H. 1954. Oxidation and Evolution of Molecular Hydrogen by Microorganisms. Bacteriol. Rev. 18:43-71.

28) Gingras, G., R.A. Goldsby, and M. Calvin. 1963. Carbon Dioxide Metabolism in Hydrogen-Adapted Scenedesmus. Arch. of Biochem. Biophys. 100:178-184.

29) Gitilitz, P.H., and A.I. Krasna. 1975. Structural and Catalytic Properties of Hydrogenase from Chromatium. Biochemistry 14:2561-2567.

30) Glick, B.R., P.Y. Wang, H. Schneider, and W.G. Martin. 1980. Identification and Partial Characterization of an Escherichia coli Mutant with Altered Hydrogenase Activity. Can. J. Biochem. 58:361-367.

31) Gogotov, I.N., N.A. Zorin, L.T. Serebriakova, and E.N. Kondratieva. 1978. The Properties of Hydrogenase from Thiocapsa roseopersicina. Biochim. et Biophys. Acta 523:335-343.

32) Gonenne, A., and R. Ernst. 1978. Solubilization of Membrane Proteins Sulfobetaines, Novel Zwitterionic Surfactants. Anal. Biochem. 87:27-38.

33) Gorrel, T.E., and R.L. Uffen. 1977. Fermentative Metabolism of Pyruvate by Rodospirillum rubrum after Anaerobic Growth in Darkness. J. Bacteriol. 131:533-543.

34) Graf, E.G., and R.K. Thauer. 1981. Hydrogenase from Methanobacterium thermoautotrophicum, a Nickel-Containing Enzyme. FEMS Lett. 136:164-171.

35) Graham, A. 1981. The Organization of Hydrogenase in the Cytoplasmic Membrane of Escherichia coli. Biochem. J. 197:283-291.

36) Graham, A., and D.H. Boxer. 1980. Arrangement of Respiratory Nitrate Reductase in the Cytoplasmic Membrane of Escherichia coli. FEBS Lett. 113:15-20.

37) Graham, A., D.H. Boxer, B.A. Haddock, M.A.M. Berthelot, and R.W. Jones. 1980. Immunochemical Analysis of the Membrane Bound Hydrogenase of Escherichia coli. FEBS Lett. 113:167-175.

38) Grande, H.J., A.V. Berkel-Arts, J. Bregh, K.V. Kijk, and C. Veeger. 1983. Kinetic Properties of Hydrogenase Isolated from Desulfovibrio vulgaris (Hildenborough). Eur. J. Biochem. 131:81-88.

39) Grande, H.J., W.R. Dunham, B. Averill, K.V. Dijk, and R.H. Sands. 1983. Electron Paramagnetic Resonance and Other Properties of Hydrogenase Isolated from Desulfovibrio vulgaris (Hildenborough) and Megasphaera elsdenii. Eur. J. Biochem. 136:201-207.

40) Gray, C.T., and H. Gest. 1965. Biological Formation of Molecular Hydrogen. Science 148:86-92.

41) Green, D.E., and L.H. Stickland. 1934. Studies on Reversible Dehydrogenase Systems. Biochem. J. 28:898-906.

42) Haddock, B.A., and C.W. Jones. 1977. Bacterial Respiration. Bacteriol. Rev. 41:47-99.

43) Hallenbeck, P.C., and J.R. Benemann. 1978. Characterization and Purification of the Reversible Hydrogenase of Anabaena cylindrica. FEBS Lett. 94:261-268.

44) Harden, A. 1901. The Chemical Action of Bacillus coli communis and Similar Organisms on Carbohydrates and Allied Compounds. J. Chem. Soc. 79:610-628.

45) Hatchikian, E.C., M. Bruschi, and J.L. Gall. 1978. Characterization of the Periplasmic Hydrogenase from Desulfovibrio gigas. Biochem. and Biophys. Res. Comm. 82:451-461.

46) Heerikhuizen, H., S.P.J. Albrecht, B. TenBrink, L.E. Twist, and E.C. Slater. 1978. Purification and Some Properties of the Soluble Part of Hydrogenase from Chromatium vinosum, in Hydrogenases: Their Catalytic Activity, Structure and Function, ed. Schlegel, H.G., and K. Schneider. E. Goltze KG, Gottingen. 151-158.

47) Hendrick, J.L., and A.J. Smith. 1968. Size and Charge Isomer Separation and Estimation of Molecular Weights of Proteins by Disc Gel Electrophoresis. Arch. of Biochem. Biophys. 126:155-164.

48) Jokoby, W.B. 1971. Enzyme Purification and Related Techniques. Methods in Enzymology, Vol. XXII, ed. Jakoby, W.B. Acad. Press. New York.

49) Jokoby, W.B. 1984. Enzyme Purification and Related Techniques Part C Methods in Enzymology, Vol. 104, ed. Jakoby, W.B. Acad. Press. New York.

50) Joklik, W.K. 1950. The Hydrogenase of E. coli in the Cell-Free State. Aus. J. Expt. Biol. Med. Sci. 28:321-329.

51) Kakuno, T., N.O. Kaplan, and M.D. Kamen. 1977. Chromatium Hydrogenase. Proc. Nat. Acad. Sci. USA. 74:861-863.

52) Karube, I., N. Urano, T. Yamada, H. Hirochika, and K. Sakaguchi. 1983. Cloning and Expression of the Hydrogenase Gene from Clostridium butyricum in Escherichia coli. FEBS Lett. 158:119-124.

53) Kelly, B.C., C.M. Meyer, C. Gandy, and P.M. Vignais. 1977. Hydrogen Recycling by Rhodopseudomonas capsulata. FEBS Lett. 81:281-287.

54) King, T.E., and R.O. Morris. 1967. Determination of Acid Labile Sulfide and Sulphydryl Groups. Methods in Enzymology, ed. Eastbrook, R.W., and M.A. Pullman. 10:634-641. Acad. Press. New York.

55) Klibanov, A.M., N.O. Kaplan, and M.D. Kamen. 1979. Chelating Agents Protect Hydrogenase Against Oxygen Inactivation. Biochim. et Biophys. Acta 547:411-416.

56) Knappe, J., J. Schact, W. Mockel, Th. Hopner, H. Vetter, Jr., and R. Edenharder. 1969. Pyruvate Formate-Lyase Reaction in Escherichia coli. Eur. J. Biochem. 11:316-327.

57) Kondo, Y., T. Kameyama, and N. Tamiya. 1957. Solubilization of the Particulate Hydrogenase. J. Biochem. (Tokyo) 44: 61-63.

58) Krasna, A.I. 1976. Oxygen-Stable Hydrogenase and Assay. Methods in Enzymol., Vol. LIII:296-301, ed. S. Fleischer and L. Packer. Acad. Press. New York.

59) Krasna, A.I. 1979. Hydrogenase: Properties and Applications. Enzyme Microb. Technol. 1:165-172.

60) Krasna, A.I. 1984. Mutants of Escherichia coli with Altered Hydrogenase Activity. J. Gen. Microbiol. 130:779-787.

61) Kruger, H.J., B.H. Huynh, P.O. Ljungdahl, A.V. Xavier, D.V. Dervartanian, I. Moura, H.D. Peck, Jr., M. Teixeira, J.J.G. Moura, and J. LeGall. 1982. Evidence for Nickel and a Three-Iron Center in the Hydrogenase of Desulfovibrio desulfuricans. J. Biol. Chem. 257:14620-14623.

62) Laemmli, U.K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227: 680-685.

63) Lall-Maharajh, V., D.O. Hall, R. Cammack, and K.K. Rao. 1983. Purification and Properties of the Membrane-Bound Hydrogenase from Desulfovibrio desulfuricans. Biochem. J. 209:445-454.

64) Lambert, G.R., and G.D. Smith. 1981. The Hydrogen Metabolism of Cyanobacteria (Blue-Green Algae). Biol. Rev. 56:589-660.

65) Lee, J.H., P. Patel, P. Sankar, and K.T. Shanmugam. 1985. Isolation and Characterization of Mutant Strains of Escherichia coli Altered in H_2 Metabolism. J. Bacteriol. 162:344-352.

66) Legall, J., D.V. Dervartanian, E. Spilker, J.P. Lee, and H.D. Peck, Jr. 1971. Evidence for the Involvement of Non-Heme Iron in the Active Site of Hydrogenase from Desulfovibrio vulgaris. Biochim. et Biophys. Acta 234: 525-530.

67) Llama, M.J., J.L. Serra, K.K. Rao, and D.O. Hall. 1981. Isolation of Two Hydrogenase Activities in Chromatium. Eur. J. Biochem. 114:89-96.

68) Lovenberg, W., B.B. Buchanan, and J.C. Rabinowitz. 1963. Studies on the Chemical Nature of Clostridial Ferredoxin. J. Biol. Chem. 238:3899-3908.

69) Macy, J., H. Kulla, and G. Gottschalk. 1976. H_2-Dependent Anaerobic Growth of Escherichia coli on L-Malate: Succinate Formation. J. Bacteriol. 125:423-428.

70) Mayhew, S.G., C. Dijk, and H.M. van der Westen. 1978. Properties of Hydrogenases from the Anaerobic Bacteria Megasphaera elsdenii and Desulfovibrio vulgaris (Hildenborough), in Hydrogenases: Their Catalytic Activity, Structure and Function, ed., Schlegel, H.G., and K. Schneider. E. Goltze KG, Gottingen. 125-140.

71) McKellar, R.C., and G.D. Sprott. 1979. Solubilization and Properties of a Particulate Hydrogenase from Methanobacterium Strain G2R. J. Bacteriol. 139:231-238.

72) Morrissey, J.H. 1981. Silver Stain for Proteins in Polyacrylamide Gels: A Modified Procedure with Enhanced Uniform Sensitivity. Anal. Biochem. 117:307-310.

73) Mortenson, L.E. 1963. Nitrogen Fixation: Role of Ferredoxin in Anaerobic Metabolism. Ann. Rev. of Microbiol. 17:115-178.

74) Mortenson, L.E. 1974. Hydrogenase. In: Microbial Iron Metabolism, ed. J.B. Neilands. Acad. Press. 231-281.

75) Mortenson, L.E. 1978. Purification and Properties of Hydrogenase from Clostridium pasteurianum. Methods in Enzymology, Vol. LIII:286-296, ed. S. Fleischer and L. Packer. Acad. Press. New York.

76) Mutaftschiev, S., M.R. O'Brian, and R.J. Maier. 1983. Hydrogen Oxidation Activity in Membranes from Rhizobium japonicum. Biochim. et Biophys. Acta 722:372-380.

77) Nakos, G., and L.E. Mortenson. 1971. Purification and Properties of Hydrogenase, an Iron Sulfur Protein, from Clostridium pasteurianum W5. Biochim. et Biophys. Acta 227:576-583.

78) Pascal, M.C., F. Casse, M. Chippaux, and M. Lepelleiter. 1975. Genetic Analysis of Mutants of Escherichia coli K-12 and Salmonella typhimurium LT2 Deficient in Hydrogenase Activity. Mol. Gen. Genet. 141:173-179.

79) Pecher, A., F. Zinoi, C. Jatisatienr, R. Wirth, H. Hennecke, and A. Bock. 1983. On the Redox Control of Synthesis of Anaerobically Induced Enzymes in Enterobacteriaceae. Arch. Microbiol. 136:131-136.

80) Peck, H.D., and H. Gest. 1956. A New Procedure for Assay of Bacterial Hydrogenases. J. Bacteriol. 71:70-78.

81) Peck, H.D., Jr., and H. Gest. 1957. Formic Dehydrogenase and the Hydrogenlyase Enzyme Complex in coli-aerogenes Bacteria. J. Bacteriol. 73:706-721.

82) Pfitzner, J., H.A.B. Linke, and H.G. Schlegel. 1970. Properties of the NAD-Specific Hydrogenase from Hydrogenomonas H16. Arch. Mikrobiol. 71:67-78.

83) Pinkwart, M., K. Schneider, and H.G. Schlegel. 1983. Purification and Properties of the Membrane-Bound Hydrogenase from N_2-Fixing Alcaligenes latus. Biochem. et Biophys. Acta 745:267-278.

84) Popov, V.O., I.V. Berezin, A.M. Zaks, I.G. Gazaryan, I.B. Utkin, and A.M. Egorov. 1983. Hydrogenase from the Hydrogen Oxidizing Bacterium Alcaligenes eutrophus Z1. Biochim. et Biophys. Acta 744:298-303.

85) Probst, I., and H.G. Schlegel. 1976. Respiratory Components and Oxidase Activities in Alcaligenes eutrophus. Biochim. et Biophys. Acta 440:412-428.

86) Rao, K.K., I.N. Gogotov, and D.O. Hall. 1978. Hydrogen Evolution by Chloroplast-Hydrogenase Systems: Improvements and Additional Observations. Biochimie 60:291-296.

87) Rao, K.K., P. Morris, and D.O. Hall. 1978. Hydrogen Evolution from Water by a Chloroplast-Hydrogenase System. In Hydrogenase: Their Catalytic Activity, Structure and Function, ed. Schlegel, H.G., and K. Schneider. E. Goltze KG, Gottingen. 141-163.

88) Sankar, P., J.H. Lee, and K.T. Shanmugam. 1985. Cloning of Hydrogenase Genes and Fine Structure Analysis of an Operon Essential for H_2 Metabolism in Escherichia coli. J. Bacteriol. 162:353-360.

89) Schink, B. 1978. Membrane Bound Hydrogenase from Alcaligenes eutrophus: Biochemical and Immunological Characterization of the Solubilized and Purified Enzyme. In Hydrogenases: Their Catalytic Activity, Structure and Function, ed. Schlegel, H.G., and K. Schneider. E. Goltze KG, Gottingen. 287-306.

90) Schink, B., and H.G. Schlegel. 1978. Hydrogen Metabolism in Aerobic Hydrogen-Oxidizing Bacteria. Biochimie 60: 297-305.

91) Schink, B., and H.G. Schlegel. 1979. The Membrane-Bound Hydrogenase of Alcaligenes eutrophus. Biochim. et Biophys. acta 567:315-324.

92) Schneider, K., and H.G. Schlegel. 1976. Purification and Properties of Soluble Hydrogenase from Alcaligenes eutrophus H16. Biochim. et Biophys. Acta 452:66-80.

93) Schneider, K., and H.G. Schlegel. 1977. Localization and Stability of Hydrogenases from Aerobic Hydrogen Bacteria. Arch. Microbiol. 112:229-238.

94) Schoenmaker, G.S., L.F. Oltmann, and A.H. Stouthamer. 1979. Purification and Properties of the Membrane-Bound Hydrogenase from Proteus mirabilis. Biochim. et Biophys. Acta 567: 511-521.

95) Sim, E., and B. Sim. 1979. Hydrodynamic Parameters of the Detergent-Solubilised Hydrogenase from Paracoccus denitrificans. Eur. J. Biochem. 97:119-126.

96) Sim, E., and P.M. Vignais. 1978. Hydrogenase Activity in Paracoccus denitrificans. Partial Purification and Interaction with the Electron Transport Chain. Biochimie 60: 307-314.

97) Sim, E., and P.M. Vignais. 1979. Comparison of the Membrane-Bound and Detergent-Solubilised Hydrogenase from Paracoccus denitrificans. Biochim. et Biophys. Acta 570:43-55.

98) Stephenson, M., and L.H. Stickland. 1930. Hydrogenase: A Bacterial Enzyme Activating Molecular Hydrogen. J. Biochimie 14:28-36.

99) Tait, R.C., K. Andersen, G. Cangelosi, and K.T. Shanmugam. 1981. Hydrogenase Genes. In Trends in the Biology of Fermentations for Fuels and Chemicals, ed. A. Hollaender et al. Plenum Press. New York. 279-303.

100) Tamiya, N., and S.L. Miller. 1963. Kinetic Studies on Hydrogenase. J. Biol. Chem. 238:2194-2199.

101) Teixeira, M., I. Moura, A.V. Xavier, D.V. Dervartanian, J. Legall, H.D. Peck, Jr., B.H. Huynh, and J.J.G. Moura. 1983. Desulfovibrio gigas Hydrogenase: Redox Properties of the Nickel and Iron-Sulfur Centers. Eur. J. Biochem. 130:481-484.

102) Thorneley, R.N.F. 1974. A Convenient Electrochemical Preparation of Reduced Methyl Viologen and a Kinetic Study of the Reaction with Oxygen Using an Anaerobic Stop-Flow Apparatus. Biochim. et Biophys. Acta 333:487-496.

103) Unden, G., R. Bocher, J. Knecht, and A. Kroger. 1982. Hydrogenase from Vibrio succinogenes, a Nickel Protein. FEBS Lett. 145:230-237.

104) Uyeda, K., and J.C. Rabinowitz. 1971. Pyruvate-Ferredoxin Oxidoreductase. J. Biol. Chem. 246:3111-3119.

105) Uyeda, K., and J.C. Rabinowitz. 1971. Pyruvate-Ferredoxin Oxidoreductase. J. of Biol. Chem. 246:3120-3125.

106) Valentine, R.C., L.E. Mortenson, and J.E. Carnahan. 1963. The Hydrogenase System of Clostridium pasteurianum. J. Biol. Chem. 238:1141-1147.

107) Van de Westen, S.G. Mayhew, and C. Veeger. 1978. Separation of Hydrogenase from Intact Cells of Desulfovibrio vulgaris. FEBS Lett. 86:122-130.

108) Van der Werf, A.N., and G. Yates. 1978. Hydrogenase from Nitrogen Fixing Azotobacter chroococcum. In Hydrogenases: Their Catalytic Activity, Structure and Function, ed. Schlegel, H.G., and K. Schneider. E. Goltze KG, Gottingen. 307-326.

109) Van Dijk, C., S.G. Mayhew, H.G. Grande, and C. Veeger. 1979. Purification and Properties of Hydrogenase from Megasphaera elsdenii. Eur. J. Biochem. 102:317-330.

110) Van'T Riet, J., A.H. Stouthamer, and R.J. Planta. 1968. Regulation of Nitrate Assimilation and Nitrate Respiration in Aerobacter aerogenes. J. Bacteriol. 96:1455-1464.

111) Viganais, P.M., M.F. Henry, Y. Berlier, and P.A. Lespinat. 1982. Effect of pH on $H-^2H$ Exchange, Hydrogen Production and Hydrogen Uptake Catalysed by the Membrane Bound Hydorgenase of Paracoccus denitrificans. Biochim. et Biophys. Acta 681:519-529.

112) Wang, R., F.P. Healey, and J. Myers. 1971. Amperometric Measurements of Hydrogen Evolution Chlamydomonas. Plant Physiol. 48:108-110.

113) Werkman, C.H. 1940. Bacterial Dissimilation of Carbohydrates. Bacteriol. Rev. 3:187-227.

114) Wilson, C.M. 1979. Studies and Critique of Amido Black 10B, Coomassie Blue R, and Fast Green FCF as Stains for Proteins after Polyacrylamide Gel Electrophoresis. Anal. Biochem. 92:63-278.

115) Winter, H.C., and D.I. Arnon. 1970. The Nitrogen Fixation System of Photosynthetic Bacteria. Biochim. et Biophys. Acta 197:170-179.

116) Yagi, T. 1970. Solubilization, Purification and Properties of Particulate Hydrogenase from Desulfovibrio vulgaris. J. Biochem. (Tokyo) 68:649-657.

117) Yagi, T., K. Kimura, H. Daidoji, F. Sakai, S. Tamura, and H. Inokuchi. 1976. Properties of Purified Hydrogenase from the Particulate Fraction of Desulfovibrio vulgaris, Miyazaki. J. Biochem. (Tokyo) 79:661-671.

118) Yamamoto, I., and M. Ishimoto. 1978. Hydrogen Dependent Growth of Escherichia coli in Anaerobic Respiration and the Presence of Hydrogenase with Different Functions. J. Biochem. (Tokyo) 84:673-679.

119) Zajic, J.E., N. Kosaric, and J.D. Brosseau. 1978. Microbial Production of Hydrogen. Adv. Biochem. Eng. 9:57-104.

120) Zeikus, J.G. 1977. The Biology of Methanogenic Bacteria. Bacteriol. Rev. 41:514-541.

121) Zinoni, F., A. Beier, A. Pecher, R. Wirth, and A. Bock. 1984. Regulation of the Synthesis of Hydrogenase (Formate Hydrogen-Lyase Linked) of E. coli. Arch. Microbiol. 139: 299-304.

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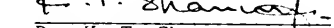

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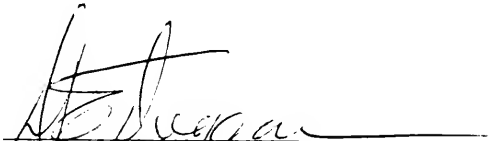

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